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Human pathogenic *Yersinia spp* are able to colonize *Steinernema* entomopathogenic nematodes

GENGLER SAMUEL

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Thèse présentée en vue de l'obtention du grade de docteur en sciences

“Friendship is the most necessary relationship for life, since no one wants to live without friends even if he had all other goods”

(*Arsitotle*)

“Persistence in scientific research leads to what I call instinct for truth.”

(Louis Pasteur)

Preface

This PhD was supported by a grant from the "Fonds pour la formation à la Recherche dans l'Industrie et dans l'Agriculture" (FRIA). The project was conducted under the supervision of Professor Henri Batoko (Group of Molecular Physiology, Institute of Life Sciences, "Université catholique de Louvain" (UCL)) and Doctor Pierre Wattiau (Foodborne Bacterial Zoonoses and Antibiotic Resistance Unit, Veterinary and Agrochemical Research Centre (CODA-CERVA)).

This thesis aims at bringing new insights in the understanding of the long-term persistence of pathogenic *Yersiniae* in the environment. Entomopathogenic nematodes (EPNs) and their potential ability to act as a biological reservoir for human pathogenic *Yersiniae* were studied in this respect.

In a general introduction we review the relevant features of the EPNs-bacteria symbiotic system and *Yersiniae*. After outlining the objectives, the results are presented into three chapters with the two first as published articles. The first chapter reports the fluorescent labelling of the bacteria of interest and their localisation inside the EPNs using epifluorescence and confocal microscopy. The second chapter describes our laboratory model explaining the colonisation and maintenance of *Y. pseudotuberculosis* inside EPNs. The third chapter presents a preliminary phenotypic analysis of three mutants of *Y. pseudotuberculosis* obtained as individual knockout of genes potentially regulating EPNs colonisation. The contribution of this work and possible perspectives are discussed in this last chapter.

Abstract

The existence of biological micro-reservoirs explaining the long-term survival of pathogenic bacteria in the environment has long been speculated. Soil invertebrates in particular are suspected to act as intermediary hosts for such pathogenic bacteria and entomopathogenic nematodes (EPNs) were investigated in this respect.

To determine whether human pathogenic *Yersiniae* are able to colonize and multiply in EPNs, a laboratory model was developed. This model consists in *Galleria mellonella* insect larvae, *Steinernema* EPNs carrying or not their natural *Xenorhabdus* symbiont and *Yersiniae*, brought artificially either in the gut of EPNs or in the haemocoele of the insect larva prior to infection. Using a single direct injection in the insect haemolymph, *Y. pseudotuberculosis* was recovered from nematodes after seven consecutive EPN infection cycles. As compared to other pathogenic *Yersiniae* as well as other pathogenic enterobacteria, *Y. pseudotuberculosis* demonstrated much higher ability to colonize *Steinernema* EPNs. Genetic determinants potentially involved in the colonization of *Steinernema* nematodes were knocked out and the resulting *Y. pseudotuberculosis* mutants were complemented with the corresponding intact genes borne on a low copy plasmid. The EPN colonization capacity of the knockout mutants could not be fully characterized for technical and timing reasons. Nevertheless, our results globally demonstrate long-term persistence of *Y. pseudotuberculosis* in EPNs in our laboratory conditions.

Finally, a method for swapping fluorescent labels in tagged bacteria was developed and used to re-engineer fluorescently labelled *Y. pseudotuberculosis.* Dual fluorescence confocal microscopic observations showed that the tagged *Yersinia* colonized distinct *Steinernema* tissues as compared to the natural *Xenorhabdus* symbiont of this nematode.

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List of Abbreviations

CAMP	cationic antimicrobial peptides
CF	Central fragment (used for overlap PCR)
CFU	Colony Forming Unit
CIP	crystalline inclusion proteins
DF	Downstream fragment (used for overlap PCR)
DNA	deoxyribonucleic acid
EPN	entomopathogenic nematode
G1	first generation
G2	second generation
GFP	Green Fluorescent Protein
Hcp	hemolysin-coregulated protein
IJ	infective juvenile
IS	Insertion Sequence
ITIS	integrated taxonomic information system
ITS	internal transcribed spacer
J1	first-stage juvenile
J2	second-stage juvenile
J3	third-stage juvenile
J4	fourth-stage juvenile
KO	knockout
LPS	lipopolysaccharides
MC	Mutagenesis cassettes
MLST	Multiple Locus Sequence Typing
NMR	Nuclear magnetic resonance
OD	Optical density
OECD	Organisation for Economic Cooperation and Development
PAAR	Pro-Ala-Ala-Arg repeat containing protein
PCR	polymerase chain reaction
PLA2	phospholipase A2
PVC	Photorhabdus virulence cassette

RAPD-PCR	random amplified polymorphic DNA PCR
RNA	ribonucleic acid
rRNA	ribosomal RNA
T3SS	Type 3 Secretion System
T3SS	type three secretion system
T6SS	Type 6 Secretion System
TAIL-PCR	Thermal asymmetric interlaced PCR
Tc	Toxin complex
TEM	Transmission Electron Microscopy
Tn	Transposon
UF	Upstream fragment (used for overlap PCR)
VgrG	valine-glycine repeat G proteins
Yop	Yersinia outer membrane proteins

.

Introduction

1. General Taxonomy of Nematodes

1.1. Nematoda and Platyhelminth

'Nematode' is the vernacular name directly derived from the phylum *Nematoda* from the Greek νῆμα (*nema*, "thread") and εἰδής (*eides*, "like"), also referred to as *Nemata* or *Nemates* (Latin plural of *nema*). The *Nematoda* phylum defined by Lankester in 1877 includes the roundworms and the horsehair worms (*Gordiidae*), while the *Nemata* phylum defined by Cobb in 1919 excludes all but roundworms from that phylum. It has been argued that *Nemata* should be the valid taxon (Luc et al., 1987). However, the Integrated Taxonomic Information System (ITIS) and a large majority of the scientific community currently recognize nematodes under the *Nematoda* phylum rather than the *Nemata*. Nematodes are described as non-segmented roundworms as opposed to segmented flat tapeworm in the *Platyhelminth* phylum. Segments in Platyhelminthes can dissociate from the main body and possess all reproductive apparatus needed to generate new worms. On the contrary, sexes in most nematodes are separated although some nematode females can be hermaphrodites. While the well-known zoonotic *Taenia saginata* or *Taenia solium* are part of the Platyhelminthes, the well-studied *Caenorhabditis elegans* or the zoonotic *Ascaris*, like *Toxocara cati* are part of the *Nematoda*. The former are called cestodes (class of *Cestoda*) while the latter are called nematodes (Class of *Secernentea*). The *Platyhelminth* phylum includes the trematodes (class of *Trematoda*) which are non-segmented flatworms.

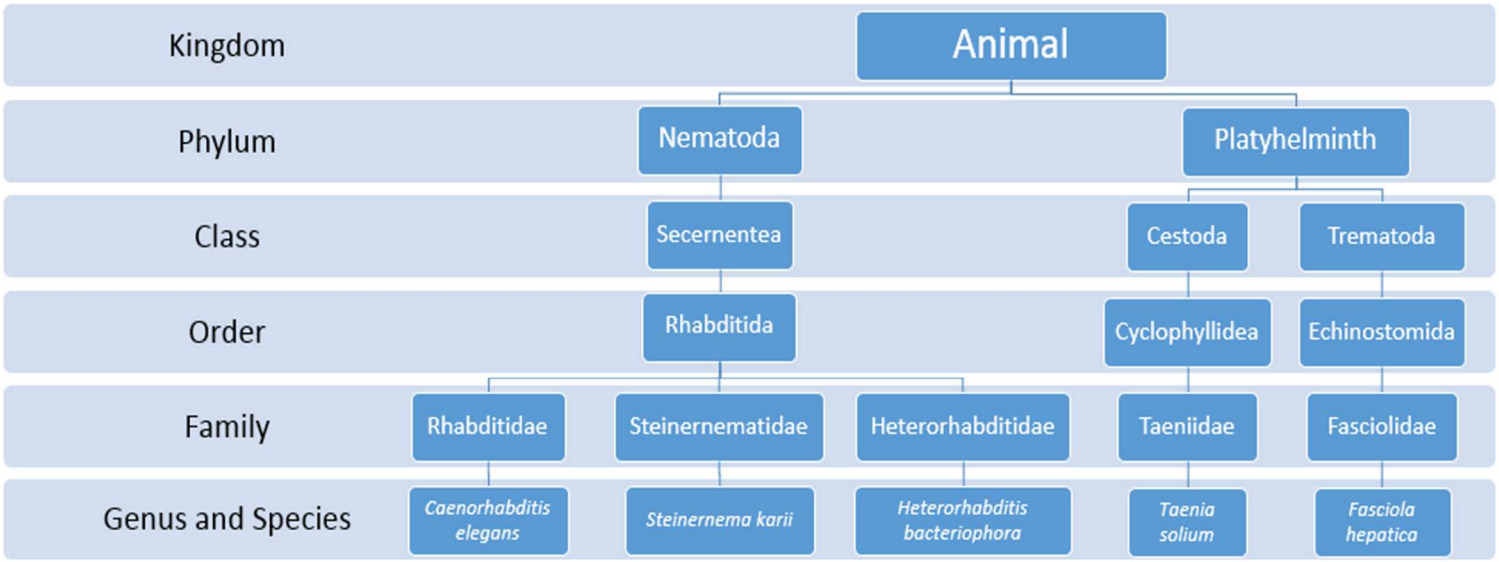

Figure 1: General simplified taxonomic tree of Nematoda and Platyhelminth

Flukes, like *Fasciola hepatica* belong to the Trematoda class. A general taxonomic tree for the *Nematoda* and *Platyhelminth* is presented in Figure 1.

1.2. Entomopathogenic Nematodes

While *C. elegans* is an exclusively free-living nematode without any parasitic stage, a huge number of nematodes alternate between a free-living stage and a parasitic stage. These nematodes include plant-pathogens as well as vertebrate and invertebrate animal pathogens.

Hereafter we will focus on insect pathogenic nematodes. These nematodes are called entomopathogenic. Entomopathogenic also comes from three Greek vocables, namely "Εντομος" (*entomos*, "insect") "πάθη" (*pathê*, "disease") and "γένος" (*guenos*, "producing"). Entomopathogenic nematodes (EPNs) are microscopic non-segmented soil roundworms exclusively feeding on insect preys. An important feature of EPNs discussed here is their partnership with an insect-pathogenic bacteria that help to to kill the insect prey. A study published in 2012 clarified the meaning of "entomopathogenicity" in the field of nematology: "EPNs must rapidly kill their hosts with the aid of bacterial partners and must pass on the associated bacteria to future generations" (Dillman et al., 2012). There are two families of EPNs, the *Steinernematidae* and the *Heterorhabditidae*. The first one contains all species of *Steinernema* (61 described up to now) and the second one contains all species of *Heterorhabditis* (14 described up to now) (Atwa, 2014). These EPNs are ubiquitous and have been found on all continents but Antarctica (Griffin et al., 1990; Kaya, 1990; Spiridonov et al., 2004).

According to Poinar the origin of *Steinernematidae* and *Heterorhabditidae* can be estimated to 375 million years ago (Poinar, 1983). Poinar postulated in 1993 that *Steinernema* and *Heterorhabditis* similarities (morphological and reproductive cycle) arose from independent convergent evolution (Poinar, 1993). Five years later, in 1998, Blaxter supported the Poinar's postulate by calculating an evolutionary tree of *Steinernematidae* and *Heterorhabditidae* based on molecular data collected by sequencing their 18S ribosomal RNA genes. This tree depicts *Heterorhabditidae* as being very closely related to *C. elegans* and *Strongylida* worms (vertebrate parasitic order of the class *Secernentea*) whilst *Steinernematidae* remain closely related to *Rhabditida* superfamilies. This tree also suggests that *Heterorhabditis* has evolved from a bactivorous nematode ancestor, making it closer to *C. elegans*, while trophic habits of *Steinernema* ancestors are ambiguous since they belong to a larger clade including plant parasitic, fungivorous and bactivorous nematodes (Blaxter et al., 1998). In other words, *Heterorhabditis* and *Steinernema* nematodes do not share an exclusive common ancestor but evolved independently from distinct ancestors according to this single locus phylogeny (Adams and Nguyen, 2002).

1.2.1. Morphological Taxonomy and Systematic

Steinernema and *Heterorhabditis* genera are very similar to each other from a morphological point of view, making them undistinguishable for a non-expert eye. However, systematic feature keys are used for the identification of EPN species (Hominick et al., 1997). The male's reproductive apparatus is one of the most discriminative feature among EPNs. The so-called spicules which are protruding from the male body can be measured (Figure 2). According

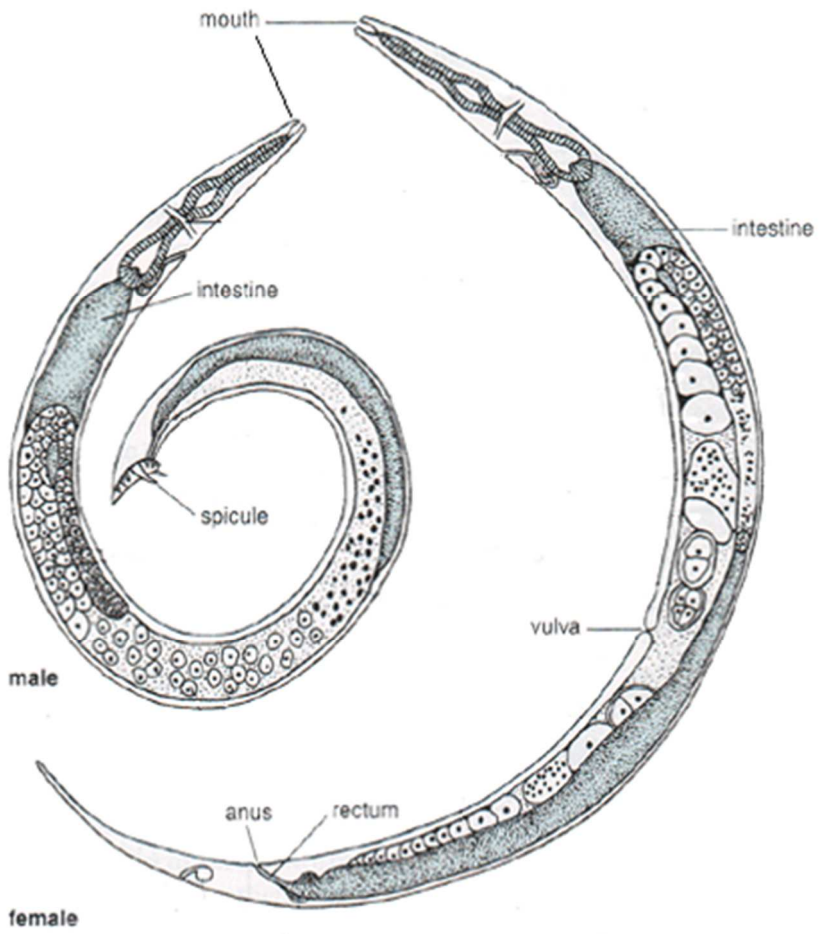

Figure 2: Schematic view of Female and male nematodes.
Figure 2 shows a transversal view of an EPN male (on the left) and an EPN female (on the right). Highlighted here, two main distinctions between EPNs males and females are the size (the females are larger) and the reproductive apparatus (spicule and vuvla). Adapted from www.studyblue.com; Accessed on February 12th 2015.

to its size and its shape it can inform on the genus and the species. The shape of the spicules allows discrimination between the first and the second male generation, giving indication on the developmental stages of the EPNs. The second generation males' spicules are more separated

from each other (Adams and Nguyen, 2002). Some measurements of females, males and third-stage juveniles (J3) EPNs allow discrimination among species as well (Nguyen and Smart, 1996), provided that the age of the J3 is taken into account. Misidentification is indeed highly probable if the harvesting time (time between description and observation of the first emerging J3) is not taken into account (Nguyen and Smart, 1995). A common feature among EPNs species is that females are always bigger and longer than males (Figure 2) although the size ratio between females and males may vary between species (Adams and Nguyen, 2002).

1.2.2. Molecular Taxonomy and Systematic

It is easily understandable that EPN identification based on morphological traits described in systematic keys can be very laborious and often requires a robust expertise. Therefore several molecular markers, partly reviewed by Liu et al. (Liu et al., 2000), have been useful to discriminate and identify EPN species. Comparison of the small ribosomal RNA (18S rRNA) nucleotide sequence allows to distinguish *Steinernematidae* from *Heterorhabditidae* (Blaxter et al., 1998; Dorris et al., 1999; Liu et al., 1997). Due to its high variability, the internal transcribed spacer (ITS) sequence lying between the 18S and 28S rRNA genes can be used to distinguish *Steinernema* and *Heterorhabditis* at the species level (Adams et al., 1998; Szalanski et al., 2000). However, ITS sequence analysis is not always sensitive enough and other molecular markers may be required for better identification. The 28S rRNA gene (Stock et al., 2001), the mitochondrial cytochrome oxidase II (COII)-16S rDNA region and the ND4 mitochondrial gene have been used for that purpose (Liu et al., 1997; Szalanski et al., 2000).

Hyman showed that divergence in mitochondrial DNA sequences can be used for speciation studies since mutations occur faster in that DNA region as compared to the genetic markers cited above (Hyman, 1988). In addition, banding patterns generated by randomly amplified polymorphic DNA PCR (RAPD-PCR) can be used to determine the genetic diversity within the same EPN species (Shapiro et al., 1997).

2. General Biology of Entomopathogenic Nematodes

2.1. Entomopathogenic nematode's life cycle

2.1.1. Life cycle overview

Steinernema and *Heterorhabditis* share similar life cycles. Both EPNs balance between a free-living stage and a parasitic stage. The free-living form of EPNs is protected from the environment by an external cuticle. Being encapsulated, the invasive EPN stage, called infective juvenile (IJ) corresponding to J3, are unable to feed because their mouth and anus are sealed (Mracek et al., 1981). They rather possess a huge lipid storage to be able to survive outside a host for several months (Selvan et al., 1994). Their lipid content depends on the lipid resources developing IJs can find in the insect host they infect. With comparable lipid reserves, it has been shown that *Steinernema* IJs survive longer in the environment than *Heterorhabditis* IJs. This can be explained by the motile behaviour of IJs. Actually a study showed that *Steinernema* IJs nictate between 50-80% of their life time while *Heterorhabditis* IJs nictate between 70-90% (Campbell and Gaugler, 1993). By doing so, lipid reserves are consumed faster in *Heterorhabditis* IJs. These observations are consistent with field experiments in which

Steinernema IJs are able to survive 24 weeks while *Heterorhabditis* IJs survive only 3 weeks in the environment (Selvan et al., 1994). Up to 20cm deep in soil, infective juveniles look for insect preys, which are most of the time insect larva (Insect adults can also be infected by EPNs (Nguyen and Smart, 1990). Once found, IJs invade the insect through natural openings such as the mouth, anus, spiracles and wounds (Grewal et al., 2001). *Heterorhabditis* IJs are also able to penetrate the insect body by directly scratching their cuticle thanks to a large anterior tooth (Bedding and Molyneux, 1982; Hill, 1998). Once inside the insect prey EPNs are able to feed and reproduce thanks to a specific bacterial symbiont they harbour. A diagram of the life cycle of Steinernema EPNs is presented in Figure 3. Upon insect penetration, IJs lose their cuticle and release the insect-pathogenic bacteria they are associated with. The couple IJs-Bacteria eventually kills the insect. IJs start feeding in the dead insect cadaver and mature into the fourth-stage juvenile (J4) which differentiates into males and females, generally 3 days post insect infestation. After mating, this first generation (G1) females lay eggs, either in the external medium or remaining in the maternal body, which will hatch into the first-stage juvenile (J1). At that point, two scenarios are possible depending on the amount of food available in the insect cadaver. In case of scarce food, J1 will mature into the second-stage juvenile (J2) within 2 or 3 days. Then J2 cease to feed and moult in pre-infective stage juvenile, also called immature IJs, before becoming infective juvenile. Then the newly generated IJ emerge from the depleted insect cadaver to actively look for another susceptible insect prey. On the contrary, if food is abundant in the cadaver, then several generations of males and females can be produced in the same cadaver. After hatching from the G1 females' eggs, J1 moult successively to J2, non-infective J3 and J4

before developing into the second generation (G2) of adults. After mating, G2 females produce eggs that will mature into J1, thereby initiating a new cycle. EPNs usually reproduce during 2 or 3 generations before total depletion of the food resources in the insect cadaver occurs (Grewal et al., 2001). The entire reproductive cycle lasts between 7 and 14 days, mainly depending on temperature, after insect invasion by IJs.

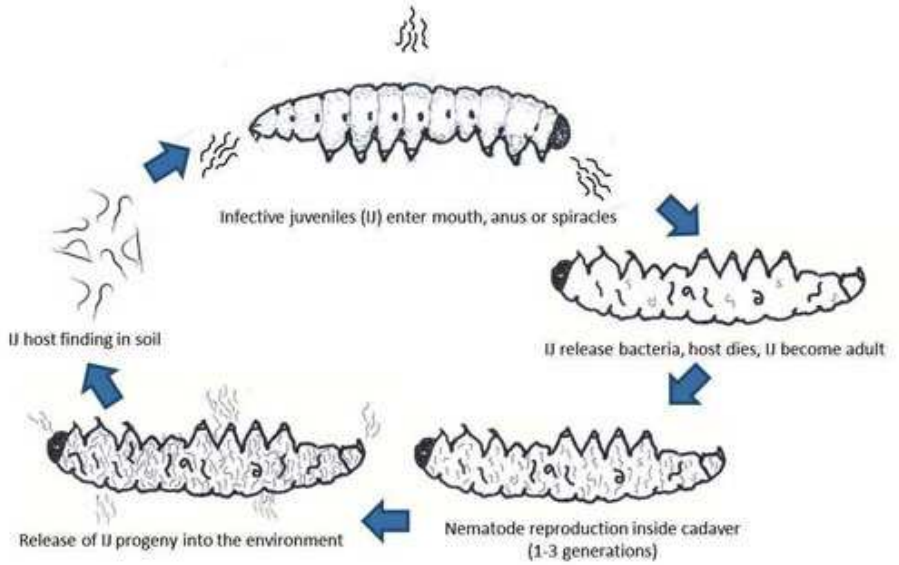

Figure 3: Diagram of the life cycle of *Steinernema*.
IJs in the soil infect an insect prey through the natural openings such as the mouth, anus or spiracles. Upon insect penetration IJs release the insect-pathogenic bacteria and the IJs-Bacteria couple eventually kills the insect. Then IJs mature into adults and nematodes start to reproduce. When the insect cadaver is depleted, IJs progeny reassociates with the bacterial symbionts and emerges from the insect looking for another insect prey. (Figure drawn by Steven Arthurs, University of Florida).

2.1.2. Entomopathogenic nematode's reproduction

Both *Steinernema* and *Heterorhabditis* females lay eggs in the insect cadaver after mating with males. Juveniles hatched from

released eggs often develop into amphimictic[1] adults (Johnigk and Ehlers, 1999a). The reproductive life cycle of most *Steinernema* involves both sexually differentiated partners, G1 males and females whilst all *Heterorhabditis* IJs develop into self-fertilizing hermaphrodite females[2] after insect infection (Poinar, 1990). However the second generation produce amphimictic *Heterorhabditis* adults. Interestingly, IJs from the species *Steinernema hermaphroditum* can develop into self-fertilizing hermaphrodite females just like *Heterorhabditis* IJs do. It has been argued that the uncommon feature of this *Steinernema* species supports the independent but convergent evolution with *Heterorhabditis* postulated by Poinar and described before (Griffin et al., 2001). As a consequence of the hermaphrodite reproduction of *Heterorhabditis* EPNs, the offspring's genetic diversity is highly decreased or impaired. The hermaphrodite behaviour of *Heterorhabditis* allows infection by a single IJ moulting into an hermaphrodite female (Hominick et al., 1996) while at least two *Steinernema* IJs have to invade an insect larva and develop into male and female. Certainly, this provides a real advantage to the survival of *Heterorhabditis* species over *Steinernema* species.

Mating between males and females consists in introducing sperm to fertilize the female's eggs. Once the vulva is found, thanks to its spicule, the male produces spermatozoids. The male's sperm will fertilize female's eggs in the uterus. For hermaphrodites self-fertilizing females, sperm is produced and stored into the spermatic vesicles

[1] sexually differentiated into males and females

[2] Hermaphrodites do possess both male and female genital tissues. However, in *Heterorhabditis* EPNs, they are called females because they exhibit similar features of females morphology (size, vulva, labial papillae, etc)

described as distal swelling of the uterus. When the female starts laying eggs, they are automatically fertilized by the sperm contained within the spermatic vesicles (Johnigk and Ehlers, 1999a; Zograf et al., 2008). Since the females are bigger and longer, males have to find a way to scan the entire female body to be able to find the vulva. There are two different ways for males to find the vulva on the female body. These two reproductive behaviours point out another distinction between *Steinernema* and *Heterorhabditis*. *Heterorhabditis* males stick to a female and slips all along the female body until it finds the vulva. Both female and male heads are pointing in opposite direction (Strauch et al., 1994). The *Steinernema* males act like a ring around the female body. The male will coil around and all along the female body until it reaches the vulva (Lewis et al., 2002).

Some mechanisms do exist to avoid several males copulating with the same female. In *Heterorhabditis* species, in analogy with *C. elegans*, the male leaves a mating plug closing the vulva after mating preventing other males to mate with the same female (Poinar et al., 1992). In *Steinernema* species, it has been shown that virgin females produce some chemical attractants for males which production decreases after mating (Lewis et al., 2002). Moreover, in order to mature *S. longicaudum* males need the presence of virgin conspecific females in their close environment (Ebssa et al., 2008).

2.1.3. Endotokia matricida

The majority of the eggs are retained inside the EPN maternal body after mating. The offspring then develops and feeds in the maternal body. This process is called *endotokia matricida* (from the Greek vocables *ενδο* ("*endo*", inside) and τοκοσ ("*tocos*", birth), and from the Latin "*mater*", mother and "*caedere*", kill). Emile Maupas

proposed the terms *endotokia matricida* when he first described *Caenorhabditis elegans* in 1900. This phenomenon has the advantage to protect the offspring and, in the case of EPNs, to provide it with a high lipidic food source especially when the infected insect cadaver is about to be exhausted (Johnigk and Ehlers, 1999b). If *endotokia matricida* is promoted in case of scarce food supply, this phenomenon occurs for the first generation of juveniles even if the food source is still abundant. It becomes then obvious that the size of the susceptible insect will affect the development and survival of EPNs. Some authors reported the inefficiency of *Steinernema* IJs to control micro-insect pests (Ebssa et al., 2004; Schroeder, 1987). Recently, Bastidas and co-workers clearly demonstrated the impaired development of EPNs in micro-insects and the complete lack of infectivity of 4 different *Steinernema* species in insects smaller than 5mm in length (Bastidas et al., 2014). From those studies came out the notion that *Steinernema,* and by inference *Heterorhabditis,* nematodes cannot persist for a long time in the environment if no larger insects are present.

2.2. EPNs as Biopesticides

2.2.1. Historical context

Both *Heterorhabditis* and *Steinernema* have the ability to cause death in a huge variety of insects (Laumond et al., 1979), making them powerful candidate biopesticides in agriculture and horticulture. In fact, the discovery and description of EPNs are intimately linked to the need of a sustainable substitute to chemical pesticides. The first EPN studied in order to develop a biological pest control agent was *Steinernema glaseri* in the early 20th century (Glaser and Farrell, 1935; Glaser and Fox, 1930; Glaser et al., 1940). But at that time chemical

pesticides were still cheap and almost no regulation existed to restrain their use. In the 1960s, more attention was devoted to the development of biopesticides when the use of chemicals became more and more restricted and unpopular. Finally in the 1980s, thanks to a huge money support and collaboration between industry and universities, researches on EPNs rapidly expanded in order to provide a very efficient control of crop pests. Nowadays studies are still conducted to identify and formulate indigenous EPN strains to improve pest control management in emerging or developing countries (Hussein and Abdel-Aty, 2012; Karimi et al., 2010; Yadav and Lalramliana, 2012).

2.2.2. Factors influencing EPNs behaviour and survival

From the 1980s EPNs were studied and engineered not only to become efficient against a broad range of insect pests, but also to be resistant to temperature variation, desiccation… Research are still ongoing and numerous parameters are checked and studied to obtain powerful biopesticides usable in different crop fields. It has been shown that temperature has an impact on the fitness and the infectivity of EPNs. High temperature first impair the ability of EPNs to infect and kill insects before eventually inducing death of EPNs (Finnegan et al., 1999; Molyneux, 1986, 1985). Low temperature as well could be detrimental to EPNs infectivity and development depending on the ability of EPNs to acclimate to a drop in temperature (Brown and Gaugler, 1996; Griffin and Downes, 1991). From *in vitro* experiments, it came out that *Steinernema* species are more cold- and hot-temperature resistant than *Heterorhabditis* species. Soil texture can significantly influence the behaviour of EPNs. It has been observed that *Heterorhabditis* are mainly found in sandy coastal soils where the salinity can be higher than in more terrestrial soils (Griffin et al., 1994).

The salinity rate of the environment as well has an impact on the infectivity of EPNs. An increased rate of salinity decreases the infectivity of *Heterorhabditis* species. On the other hand, salinity improves the hot-temperature tolerance of *Heterorhabditis* (Finnegan et al., 1999). From these studies it has been observed that each EPNs species behaves differently when faced to temperature shifts or suboptimal temperatures. These examples illustrate the high number of parameters which can impact the infectivity and survival of EPNs, making it very difficult to generalise the environmental behaviour of EPNs (Griffin, 2012). This variability explains why EPN species used as biopesticides will vary according to the targeted pest and the geographical localization of the targeted crop. Moreover, the idea of introducing non-indigenous EPNs might not be well-accepted and the impact on the non-targeted host should be evaluated. Bathon discussed these issues and conclude that the impact on the autochthon non-targeted fauna is negligible since the population could be only affected over a space- and time-restricted scale (Bathon, 1996). These assumptions will have to be reconsidered if EPN biopesticides should be generalised. In 1995, the Organisation for Economic Cooperation and Development (OECD) reviewed the issue of introduction non-indigenous nematodes for biological control. The OECD report concluded that scientific evidence supports the assumption that EPNs are safe and that the few existing risks were identified. Moreover, the report noted that EPNs "are more specific and are less of a threat to the environment than chemical pesticides" (Ehlers and Hokkanen, 1996).

Despite the impact on the non-targeted fauna, it should be wise to check if these EPNs largely spread over cultures, might carry and/or take up some mammal and human bacterial pathogens. Up to now, no study has been conducted to deal with this issue. First because the

microbial content of EPNs used as biopesticide is strictly controlled. Indeed during the industrial production process, EPNs go through an axenic step, before being used on the field (Bedding, 1981; Friedman et al., 1990). Second, no human or mammal infection strictly due to EPNs has ever been recorded. EPN strains used as biopesticide revealed to be non-pathogenic for humans so far. Nevertheless, the possible ability of EPNs to take up pathogens naturally present in the environment has to be addressed.

3. Bacterial Symbiont of Entomopathogenic Nematodes

Numerous organisms are living in symbiotic partnership, especially insects and bacteria (Moya et al., 2008). A very strong mutualistic association exists between lots of aphids and their intracellular endosymbiont *Buchnera aphidicola* located inside specialized host cells called bacteriocytes (Belda et al., 2010). Interestingly, in the aphid *Cinara cedri*, a second symbiont, *Serratia symbiotica*, is needed since *B. aphidicola* is not sufficient to fulfil all symbiotic functions (Burke and Moran, 2011; Manzano-Marín et al., 2012). The tsetse fly (Diptera: Glossinidae) is also symbiotically associated with several bacteria like *Wigglesworthia glossinidia* and *Sodalis glossinidius* (Aksoy, 1995; Belda et al., 2010). *Onchocerca volvulus* is a parasitic filarial nematode causing skin and eye pathology called onchocerciasis in human. This nematode harbours an intracellular obligate bacterial symbiont called *Wolbachia*, which is essential for its normal development and fertility (Brattig, 2004; Taylor et al., 2005). It appeared that *Wolbachia* rather than the nematode is the cause of human inflammations (Gillette-Ferguson et al., 2006).

Entomopathogenic nematodes also have the particularity to live in symbiosis with specific bacteria. The genus to which these bacterial symbionts belong differs between *Steinernematidae* and *Heterorhabditidae*. The former are always associated with *Xenorhabdus* species, while the latter are associated with *Photorhabdus* species (Forst and Nealson, 1996; Poinar, 1990). In contrast to their respective nematode hosts, *Xenorhabdus* and *Photorhabdus* genera are closely related from a phylogenetic point of view. Actually they are more closely related to each other than to any other bacterial genera (Adams et al., 2006).

More details and descriptions on the roles of the bacterial symbionts in the life cycle of EPNs are given hereafter.

3.1. General taxonomy of Photorhabdus spp. and Xenorhabdus spp.

3.1.1. Historical discovery

In 1955, Dutky and Hough found an undescribed insect parasitic nematode (DD-136) causing death to *Carpocapsa pomonella*. For the first time, these authors stated that the death of the insect larva was due to bacterial septicaemia (Dutky and Hough, 1955). Intrigued by this statement, Poinar and Thomas made a more in depth study of this DD-136 EPN (later shown to belong to the *Steinernematidae*). In 1965, they discovered the recurrent association between the *Steinernematidae* DD-136 and a specific bacterium which was described as *Achromobacter nematophilus* sp. nov. since the authors were unable to classify it (Poinar and Thomas, 1965). One year later, the same authors described the mutualistic relationship between the nematode and its bacterial symbiont (Poinar and Thomas, 1966). In 1979, the new genus

Xenorhabdus gen. nov. was proposed to gather all the mutualistic bacteria associated with EPNs. Despite some biochemical and physiological differences, no genus distinction was made between bacterial symbionts of *Steinernema* and *Heterorhabditis* nematodes. However it has been observed that bioluminescent *Xenorhabdus* sp. are always associated with *Heterorhabditis* nematodes but never with *Steinernema* nematodes (Thomas and Poinar, 1979). With the development of molecular tools, based on DNA relatedness analysis, Boemare et al. proposed in 1993 the new genus *Photorhabdus* gen. nov. to term the bacterial symbiont of *Heterorhabditis* nematodes (Boemare et al., 1993). However, in 1977, a *Photorhabdus* species, named *Photorhabdus asymbiotica* (Fischer-Le Saux et al., 1999) (formerly *Xenorhabdus luminescens* from DNA hybridization group 5 (four strains)) was found to infect humans as an opportunistic agent. It was isolated from leg wounds and blood sample of clinical patients in the USA without any *Heterorhabditis* EPNs (Farmer et al. 1989). The natural *Heterorhabditis* nematode host was identified in 2006 from soil samples which caused infection by *P. asymbiotica* of a 49-year old Australian man who dug to build a fence (Gerrard et al., 2006). Up to now, *P. asymbiotica* is the only EPN bacterial symbiont known to be directly pathogenic towards mammalian host.

3.1.2. Taxonomy

Both *Xenorhabdus* and *Photorhabdus* are gram negative asporogenous rod-bacteria members of the *Enterobacteriaceae* family (Boemare and Akhurst, 1988; Boemare et al., 1993). They are motile with peritrichous flagella (Akhurst & Boemare 1988; Farmer et al. 1989). They are facultatively anaerobic with both respiratory and fermentative metabolism (Boemare, 2002). As mentioned before,

bioluminescence of *Photorhabdus* bacteria is one of the most obvious characteristic they do not share with *Xenorhabdus* symbionts. They are all entomopathogenic with a host spectrum varying among species. For example, the couples *S. carpocapsae-X. nematophila* and *H. bacteriophora-P. luminescens* are highly effective against a broad range of insects from *Lepidoptera*, *Coleaptera* and *Orthoptera* orders (Fallon et al., 2006; Rosa et al., 2002; Wang et al., 1994). On the opposite the couple *S. glaseri-X. poinarii* is virulent only to a few coleopteran species (Converse and Grewall, 1998). *X. poinarii* injected alone in *G. mellonella* larvae (a highly susceptible Lepidoptera), is unable to kill the larvae while other *Xenorhabdus* species are (Akhurst, 1986). This narrow host specificity clearly suggests peculiar adaptation of *X. poinarii* to its nematode host. The suggested evolutionary pathway of *X. poinarii* is supported by its smaller genome size compared to other *Xenorhabdus* symbionts (Ogier et al., 2014). Both EPN symbiont genera produce bacteriocins which will be described later in this introduction. It is interesting to note here that many strains of *Xenorhabdus* are lysogenic while no phage has yet been found in *Photorhabdus* (Boemare et al., 1992; Forst et al., 1997). A phage-tail like bacteriocin, called xenorhabdicin, has been purified from *X. nematophila* (formerly *X. nematophilus*). This bacteriocin showed to be effective against closely related bacteria like *Photorhabdus* and other *Xenorhabdus* species (Boemare et al., 1992; Thaler et al., 1995).

3.1.3. Phenotypic variation

In general, pathogenic bacteria use phenotypic variation to respond to environmental changes (Robertson and Meyer, 1992). This phenotypical switch does not occur in the entire bacterial population and a clonal population issuing from a single bacterium is never fully

homogenous from a genetic point of view. This is a protective mechanism for a better survival whatever the change in the growth conditions (Forst et al., 1997). Both *Xenorhabdus* and *Photorhabdus* genera display phenotypic variation. Several stress conditions have been identified that enhance the phenotypic shift such as nutriment depletion (Smigielski et al., 1994) and microaerophilic or anaerobic conditions (Boemare and Akhurst, 1990). There is two phenotypic profiles in EPNs symbionts described as Form I and Form II variants. Form I bacterial variants are isolated from the IJ stage of EPNs' life cycle (Boemare and Akhurst, 1988). Form II variants either lack or have modified biochemical properties (see Boemare & Akhurst 1988) such as bromothymol blue dye adsorption - form I variants do absorb the dye while form II variants do not (Akhurst, 1980). Form II variants of *P. luminescens* exhibit very weak or no bioluminescence at all (100-fold greater in form I) (Boemare and Akhurst, 1988; Fischer-Le Saux et al., 1999). Both *Xenorhabdus* and *Photorhabdus* display a severe decrease in the production of antibiotic molecules and crystal proteins needed for the proper development of their nematode host inside an insect prey (further described in the text). It has been shown that both variant I and II are equally pathogenic towards *G. mellonella*. However, if entomopathogenicity of the bacterial symbiont is not affected by phenotypic variation, the number of IJs emerging from a *G. mellonella* larva infected with form II symbionts is drastically reduced as compared to a larva infected with form I symbionts (Volgyi et al., 1998). Form I symbionts are motile and able to swarm on the LB agar surface while form II variants are not motile due to the absence of expression of the flagellar protein encoding genes *fliCD* (Givaudan et al., 1995). Thanks to transmission electron microscopy (TEM), Brehélin and co-workers showed that the capsular material of *Photorhabdus* and

Xenorhabdus symbionts is also affected by phenotypic variation. If *P. luminescens* harbour fimbriae in both form I and II, *X. nematophila* cell surface loses all its fimbriae in form II. The glycocalyx layer, though thicker in *Xenorhabdus* than in *Photorhabdus*, is significantly thinner in both symbiont form II variants (Brehélin et al., 1993).

Phenotypic variation does not imply molecular changes in DNA neither in *Xenorhabdus* nor in *Photorhabdus*. This is supported by several observations. First, DNA relatedness between form I and form II variants is 100% (Boemare et al., 1993). Second, digestion profiles of genes encoding proteins affected by phenotypic variation (flagella, bioluminescence, crystal proteins …) are identical in form I and form II. Southern cross experiments also demonstrated no DNA rearrangement (Akhurst et al., 1992). The plasmid content of both phenotypic variant was also found to be the same (Leclerc and Boemarel, 1991; Smigielski and Akhurst, 1994). Finally, Givaudan and co-workers found that *Xenorhabdus fliCD* flagellar genes are intact but not expressed in form II *Xenorhabdus* variants. The motile phenotype is indeed restored in a complemented *E. coli* flagellin mutant while complementation of the form II variant with the same *fliCD* genes does not restore the motile phenotype in *Xenorhabdus*. The same authors showed that the nucleotide sequence of *fliCD* and of the surrounding region was identical in both form I and form II variants but Northern blot analysis demonstrated that *fliC* and *fliD* are not and weakly transcribed respectively (Givaudan et al., 1996). If DNA instability is not involved in phenotypic variation, several experiments demonstrated regulation of phenotypic variant genes at the RNA level (transcriptional (*fliCD*) or post-transcriptional (*lux*) levels) or at post-translational (lipases and proteases) level (see Forst et al. 1997 for review).

Reversions (from form II to form I) are possible but happen infrequently in *Xenorhabdus* and was so far never reported in *Photorhabdus* (Forst and Clarke, 2002; Joyce et al., 2006). For *P. asymbiotica,* form II variants were recovered from human clinical samples (Fischer-Le Saux et al., 1999). However Gerrard and co-workers showed that *P. asymbiotica* (Kingscliff strain) recovered from a human hand wound appeared to be a form I variant since it was able to support the development of its *Heterorhabditis* host just as well as a *P. asymbiotica* strain directly isolated from the nematode (Gerrard et al., 2006). This suggests that *Photorhabdus* spp. are also able to revert from form II to form I.

3.2. Bacterial symbionts along EPN life cycle

The symbiotic relationship between *Xenorhabdus* and *Photorhabdus* symbionts with their respective nematode hosts is cyclic and balanced along the life cycle of EPNs between a symbiotic and a feeding stage. In analogy with section 2.1.1., we will start the cycle description from the IJs stage.

3.2.1. *Photorhabdus* and *Xenorhabdus* hosted in IJs

Symbiotic bacteria are hosted in the gastro - intestinal tract of IJs released in the environment when they actively seek for an insect prey. *Xenorhabdus* bacteria are more specifically located in an intestinal vesicle, originally called symbiotic vesicle (Bird and Akhurst, 1983) but currently described as receptacle (Snyder et al., 2007). This receptacle is located between the pharynx and the intestine (Figure 4). The size and morphological variability of the receptacle has been shown by Bird and Akhurst thanks to differential interference contrast microscopy and TEM. It appeared that the thickness of the receptacle wall varied

from a *Steinernema* species to another, being thick in *S. kraussei* and thin in *S. feltiae*. Some receptacle's lumens were covered with microvilli.

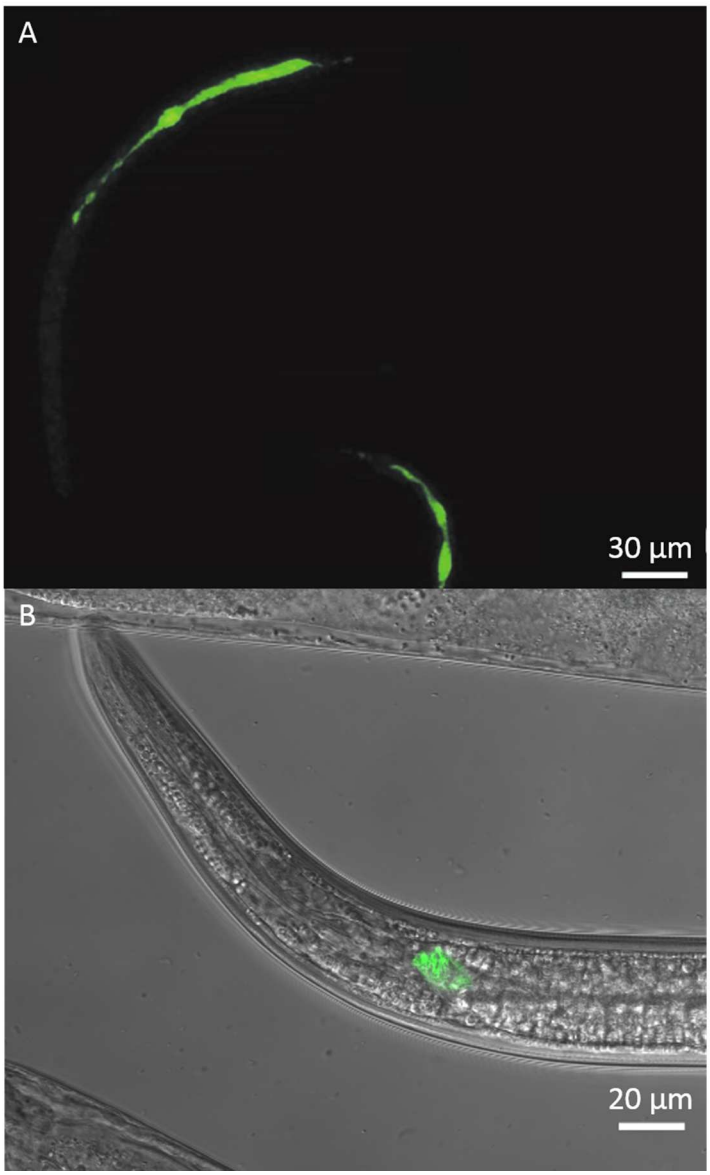

Figure 4: EPNs colonized by GFP-labelled symbiotic bacteria

A. Epifluorescent micrograph showing the location of GFP-labelled *P. luminescens* bacteria in the intestines of *H. bacteriophora* IJs (Ciche and Ensign, 2003).

B. Confocal micrograph showing the location of *Xenorahbdus* sp. TZ03 in the receptacle of a *Steinernema* sp. MW8B IJ (Gengler et al., 2015).

The same authors also showed that development of the receptacle is independent of the presence of *Xenorhabdus* cells. Actually similar but empty structures developing in axenic *S. bibionis* were observed (Bird and Akhurst, 1983). However, the presence of *Xenorhabdus* inside the receptacle influences its structure since it is shorter and wider in axenic IJs (Flores-Lara et al., 2007). A more recent study confirmed the previous statement and went deeper in the analysis of the receptacle structure allowing a finer classification of Steinernematidae (Kim et al., 2012). A *Steinernema* IJ usually carries between 40 and 200 *Xenorhabdus* CFUs (Martens et al., 2003; Poinar, 1966). *Heterorhabditis* nematodes do not possess a symbiotic vesicle. As a result, an average of 130 CFUs of *Photorhabdus* symbionts are mainly located in the anterior part of the nematode digestive tract together with other Enterobacteriaceae (Figure 4) (Babic et al., 2000; Ciche and Ensign, 2003; Endo and Nickle, 1991; Milstead, 1979). Each EPN has its specific bacterial symbiont even if bacterial symbionts taxonomically attributable to the same bacterial species can be symbiotically associated with several EPN species. Significant differences do exist between different isolates of the same *Xenorhabdus* species, though, like sugar assimilation or growth temperature. These differences are believed to reflect specific adaptations to their dedicated *Steinernema* host (Emelianoff et al., 2008b; Tailliez et al., 2006). Such nematode-bacterium specificity is also observed in *Heterorhabditis* spp. since they cannot reproduce when fed with another *Photorhabdus* symbiont than their natural one (Han and Ehlers, 1998). Even if they can be cultivated as free organisms in vitro, bacterial symbionts are usually never retrieved from the environment outside their EPN hosts.

3.2.2. Insect invasion and release of bacterial symbionts

Upon invasion of an insect larva, IJs release their bacterial symbionts by excreting (in the case of *Xenorhabdus*) or regurgitating (in the case of *Photorhabdus*) them directly in the insect's haemocoele (Balcerzak, 1991; Ciche and Ensign, 2003; Poinar and Himsworth, 1967). It has been shown that the insect haemolymph, and not the presence of nutrients, triggers the release of either *Xenorhabdus* or *Photorhabdus*. Moreover, it has been demonstrated that bacterial motility is not required for symbiont release out of the IJ body. The nematode thus controls the release of its bacterial symbiont (Ciche and Ensign, 2003; Snyder et al., 2007). It is interesting to note here that from that particular moment, bacterial symbionts and nematode hosts are, in close proximity, although existing separately (Stock and Goodrich Blair, 2008). Bacterial symbionts shift to a dramatically different environment, moving from the nematode's gut to the insect haemolymph. The global regulator Lrp is essential to activate or represse gene involved either in mutualism (colonization factors like *nilABC* described further in the text) or parasitism (insect virulence factors) according to the bacterial environment (Cowles et al., 2007). A signal for bacterial symbionts to adapt to their new environment is the presence of high iron concentration in the insect as compared to the IJs gut. Iron concentration acts on the expression of genes under the control of the FliAZ regulon which encode proteins involved in mutualism and pathogenicity (exoenzymes, full virulence toward insects, motility) (Joyce et al., 2006; Jubelin et al., 2011; Lanois et al., 2008; Watson et al., 2010, 2005). FliA is a sigma factor notaby required for the expression of the flagellin monomer FliC (Givaudan and Lanois, 2000). FliZ regulates the expression of two haemolysin genes involved in apoptotic and pore-forming activities in insect cells

(Vigneux et al., 2007; Zhang et al., 2014). Jubelin and co-workers demonstrate that a low availability of iron downregulates the expression of the genes under the control of the FliAZ regulon (Jubelin et al., 2011). In the insect's haemolymph, symbionts rapidly multiply causing a fatal septicaemia to the infected insect. Bacterial symbionts also produce insecticidal toxins helping killing the insect host and antibiotic compounds preventing competitors development in the insect cadaver (Akhurst, 1993; Sicard et al., 2003; Nick R Waterfield et al., 2009). The insecticidal toxins and the molecules secreted to prevent competition inside the cadaver will be described in the following sections. As described before, after symbiont release, nematodes start to feed and reproduce. Their food supply is provided by the symbiont which degrades the insect's macromolecules. In addition, bacterial symbionts themselves serve as food source for nematodes as well (Chaston et al., 2011). *Xenorhabdus* and *Photorhabdus* bacteria massively produce cytoplasmic crystalline inclusion proteins (CIP) (named IP-1 and IP-2 in *Xenorhabdus* and CipA and CipB in *Photorhabdus*) which may serve as food source for nematodes. *Xenorhabdus* or *Photorhabdus* CIP knock-out (KO) mutants cannot therefore support multiplication of nematodes in vivo (Bintrim and Ensign, 1998; Couche and Gregson, 1987). However, it has been observed that nematodes cannot multiply when grazed on killed symbiont containing the inclusion proteins. This observation suggest a more complex function for these inclusion proteins probably involved in the nematode symbiosis (Bowen and Ensign, 2001). CIP accounts for up to 40% of the *Photorhabdus* protein content and for more than one third of the total cell volume of *Xenorhabdus* (Bowen and Ensign, 1998; Couche and Gregson, 1987). The IP-1/-2 are composed of a single protein subunit with a molecular mass of 26 and 22 kDa respectively while the molecular mass of the

CipA/B protein subunit is 11 kDa. The high content of hydrophobic amino acids of these inclusion proteins may explain their insolubility at the neutral pH of the *Xenorhabdus* and *Photorhabdus* cytoplasm (Bowen and Ensign, 2001). If they have 25% similarity with each other, the peptidic sequences of these inclusion proteins do not share similarity with any other known protein so far (Bintrim and Ensign, 1998). It is then obvious that more research has to be done to determin more precisely the function of these proteins.

3.2.3. Colonization of new IJ generation by the bacterial symbiont before emergence.

When the insect cadaver is depleted, EPNs stop multiplying and larval maturation stops at the IJ stage (see section 2.1.1.). Before emerging from the insect cadaver, IJs and their specific symbionts have to re-associate to ensure successful future infections and survival of both partners. The developing IJs then have to cease feeding on bacterial symbionts to allow them to colonize their gut. In the meantime, EPNs symbionts have to switch from a high insect pathogen to a harmless IJ colonizator. Colonization of IJs differs from *Xenorhabdus* to *Photorhabdus*. For the former, symbionts are transmitted horizontally in *Steinernema* while they are vertically transmitted through the maternal body of *Heterorhabditis* for the latter. The presence of a receptacle in *Steinernema* nematodes and its absence in *Heterorhabditis* nematodes, makes the bacterial symbionts confined in *Steinernema* IJs while *Photorhabdus* are rather scattered along the *Heterorhabditis'* gut.

3.2.3.1. Colonization of Steinernema by Xenorhabdus

In 2003, Martens and co-workers were the first to get through an in-depth analysis of the colonization of *S. carpocapsae* IJs by *X. nematophila*. With the help of GFP mutants of *X. nematophila*, they demonstrated that a few bacterial cells initiated colonization of the receptacle (qualified as oligocolonization by the authors). By combining epifluorescence microscopy pictures with symbiont CFUs counting in oligocolonized pre-infective juvenile during 150h, they showed that bacteria multiply in the receptacle's lumen to reach full density, since mouth and anus are already sealed in immature IJs. Finally the same authors, using three different signature-tagged *X. nematophila* clones, demonstrated that the *S. carpocapsae* receptacle is most of the time (75%) colonized by a single *X. nematophila* clone supporting the oligocolonization and the high specificity of the Nematode-Bacteria symbiosis (Martens et al., 2003). Two years later, the same laboratory discovered the presence of a so-called 'intravesicular structure' (IVS) inside the receptacle. They described the IVS as an untethered cluster of anucleated spherical bodies which are not attached to the receptacle epithelium. The true nature of these IVS is still undetermined, but spheres might be surrounded by a mucus-like layer which may serves as specific binding site for *Xenorhabdus*. Indeed, authors observed the binding of *Xenorhabdus* to the IVS in the early stage of colonization. As for the receptacle, the presence of *Xenorhabdus* is not required for the formation of the IVS. Authors showed that insect hemolymph triggers the release of IVS from the receptacle and its excretion with or without binding *Xenorhabdus* cells. Clearly the IVS does not only mediate specific colonization of the *Steinernema* receptacle by *Xenorhabdus* bacteria but also helps bacterial release upon insect infection. (Martens and Goodrich-Blair, 2005). Some determinants have been identified to be

involved or required for the colonization of *Steinernema* by *Xenorhabdus*. Defective metabolic mutant unable to synthesize methionine or threonine were able to initiate the colonization but were unable to grow within the receptacle (Flores-Lara et al., 2007; Martens and Goodrich-Blair, 2005). Also mutants lacking the RpoS sigma factor were not at all able to colonize the nematode (Heungens et al., 2002; Vivas and Goodrich-blair, 2001). The *nilABC* genes (for nematode intestine localization), coding for membrane proteins involved in adherence, are necessary for the colonization of *S. carpocapsae* nematodes by *X. nematophila*. Besides being unable to colonize the nematode's gut, *nil* KO mutants display no other essential phenotype alterations such as entomopathogenicity or support to nematodes multiplication (Heungens et al., 2002). Sicard and co-workers studied the species-specific symbiosis between *X. nematophila* and *S. carpocapsae* (Sicard et al., 2004). Interestingly, *X. nematophila nil* locus homologs cannot be found in any other *Xenorhabdus* species described up to now. Introduction of the *nil* locus in other *Xenorhabdus* species allow them to colonize *S. carpocapsae* while wild-types are unable. All together, these observations demonstrate not only the species-specificity of *S. carpocapsae* colonization but also the essential role of the *nil* locus in this phenomenon (Cowles and Goodrich-Blair, 2008). Lately, thanks to gfp-tagged bacteria, Chaston and co-workers showed the localization of *X. nematophila* through the all developmental stages of its nematode host *S. carpocapsae* thus demonstrating that the symbiont is not only associated to the juvenile stage (Chaston et al., 2013). Figure 5 shows the different localisation (either in the intestinal tract, or in the receptacle or in pharyngeal-intestinal valve) of *X. nematophila* through the development of *S. carpocapsae*. Other studies showed that the CpxRA signal transduction system is an important

regulator of *Xenorhabdus* behaviour, either promoting mutualism with a *Steinernema* nematode (regulation of the *nil* locus) or promoting virulence toward insects (resistance to insect immune system) (Herbert et al., 2007; Herbert Tran and Goodrich-Blair, 2009; Herbert Tran et al., 2009).

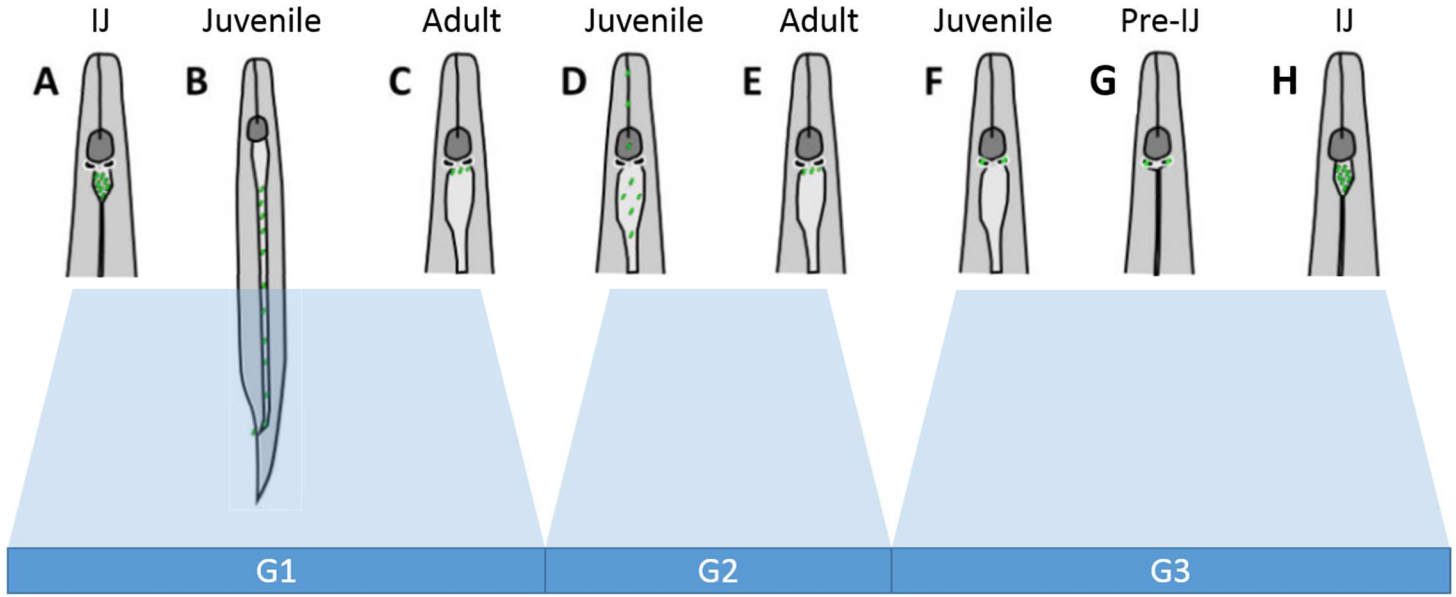

Figure 5: Spatial and temporal *X. nematophila* colonization events during *S. carpocapsae* development

(A) A colonized infective juvenile (IJ) (B) releases its bacteria by defecation after insect penetration and moults into adult stages. (C) Adults that develop from IJs carry bacteria localized at the anterior intestinal caecum. (D) Bacteria localize randomly through the intestin. (E) G2 adult nematodes have bacteria localized to the anterior intestinal caecum. The beginning of pre-IJ development is distinguished by (F) bacterial colonization of the pharyngeal-intestinal valves, and subsequently, intestinal constriction (G). (H) After relaxation of the anterior intestinal constriction, bacteria are observed localized to the receptacle and over time bacteria grow to completely fill the receptacle. Abbreviations: parental generation (G1), first generation offspring (G2), second-generation offspring (G3). (Adapted form Chaston et al., 2013)

3.2.3.2. Colonization of Heterorhabditis by Photorhabdus

Despite the absence of receptacle in *Heterorhabditis*, species-specific colonization of *Heterorhabditis* by *Photorhabdus* exists and can be even more stringent than in *Steinernema*/*Xenorhabdus* couples. *Heterorhabditis* IJs are formed by endotokia matricida. Consequently, transmission of *Photorhabdus* to IJs is maternal and thus vertical. Three major steps are described for IJs colonization. The first one is the migration of *Photorhabdus* towards the rectum of the hermaphrodite females. Second, colonization of the anterior part of IJ's gut occurs during the endotokia matricida. And finally, the first colonizing bacteria multiply and reach the whole IJ's gut (Ciche and Ensign, 2003; Goodrich-Blair and Clarke, 2007). In 2008, Ciche and co-workers led an in-depth study to shed light on the mechanisms of *Photorhabdus* transmission. Thanks to GFP-labelled bacteria and transmission electron microscopy (TEM) observations, they showed that after biofilm-mediated adherence and colonization of the hermaphrodite's gut, *Photorhabdus* cells migrate to and invade the rectal glands. Within this compartments, bacterial symbionts are available for offspring which hatches inside the maternal body (Ciche et al., 2008). Another study published in 2012 and making use of the same methods as that of Ciche et al., argued that *Photorhabdus* cells do not invade rectal glands in the hermaphrodites, but rather vesicles in the rectum area (Stock et al., 2012). The true mechanism is still not clearly established but everybody agrees on the maternal transmission of *Photorhabdus* cells into IJs, which differs strikingly with the horizontally transferred *Xenorhabdus* cells. Despite the fact that *Heterorhabditis* sp. do not possess any receptacle, high selective processes exist to favour colonization by the bacterial symbiont. This process is partially

dictated by the innate immune response of the nematodes themselves. Actually hermaphrodites produce some CAMP-like (cationic antimicrobial peptides) proteins to which *Photorhabdus* bacteria might resist thanks to the product of the *pbgPE* operon involved in adaptive lipopolysaccharide (LPS) modifications (Froy, 2005; Goodrich-Blair and Clarke, 2007).

3.3. Entomopathogenicity of Xenorhabdus spp. and Photorhabdus spp.

Pathogenicity of *Xenorhabdus* and *Photorhabdus* towards insects can be summarized in two aspects. The first aspect consists in overcoming the insect immune response. The second aspect consists in releasing a toxins to kill insects.

As mentioned before, the two EPNs symbiotic bacterial genera are genetically closely related but behave differently to achieve their symbiotic lifestyle. The way the insect immune response is overcomed perfectly illustrates the convergent evolution of *Xenorhabdus* and *Photorhabdus* bacteria. It has been shown that pre-injection with non-pathogenic bacteria (*E. coli*) up-regulates the innate immune response of *Manduca sexta* and confers to the insect a significant general resistance to enterobacteria including *Xenorhabdus* and *Photorhabdus* (Eleftherianos et al., 2006). Like mammals, insects produce some CAMPs targeting the membranes permeability of both Gram-negative and –positive bacteria. There is a huge diversity of CAMPs thus displaying different effect on the targeted bacteria. Some CAMPs like proline-riche peptides (Buforin II, dermaseptin, indolicin) have a cytotoxic activity bloking DNA replication and transcription. They either bind DNA/RNA or induce degradation of essential enzyme for DNA replication resulting to an inhibition of both DNA replication

and transcription (Brogden, 2005; Bulet et al., 1999; Nappi and Ottaviani, 2000). Some other CAMPs recognize the general structure of the lipopolysaccharide (LPS) shared by Gram-negative bacteria. Thus to counteract this humoral response, *Photorhabdus* expresses a modified LPS which hinders the effect of CAMPs by preventing the recognition of the new LPS structure and decreasing the outer-membrane's permeability to these compounds (Guo et al., 1998; Medzhitov and Janeway, 1997). This strategy is quite common among enterobacteria. For example, *Salmonella* adds a sugar to its LPS variable part thanks to the products of the *pmr* operon which is regulated by the *phoPQ* locus (Gunn et al., 1998; Guo, 1997). The *phoPQ* operon as well as *pbg*, a homolog of the *pmr* operon, are present in the *Photorhabdus* genome. As mentioned before, *pbgE* KO mutants are unable to colonize *Heterorhabditis* IJs as the probable consequence of their hyper-susceptibility to CAMPs produced by hermaphrodites. It has been shown that this mutant fails to kill the insect as well due to its inability to overcome the insect's humoral response (Bennett and Clarke, 2005). More largely, Derzelle and co-workers showed that the *Photorhabdus phoP* KO mutant was also unable to kill insects (Derzelle et al., 2004). Interestingly, the same mutants in *Xenorhabdus* are also more susceptible to CAMP, although they are still able to kill insects (Goodrich-Blair and Clarke, 2007). This means that *Xenorhabdus* can counteract the insect humoral response but does so through another strategy as compared to *Photorhabdus*. In addition to LPS modification, *Xenorhabdus* prevents the expression of insects CAMP genes (Ji and Kim, 2004). Insects trigger a cellular immune response which involves haemocytes able to aggregate, encapsulate and melanise bacteria. By doing so a nodule containing trapped bacteria is formed and removed from hemolymph circulation (Kanost et al., 2004). *Xenorhabdus* and

Photorhabdus bacteria can secrete an inhibitor of the insect's phospholipase A2 (PLA2), involved in melanisation and nodule formation. Thus, by inhibiting PLA2, *Xenorhabdus* and *Photorhabdus* escape from this part of the insect cellular immune response (Kim et al., 2005; Shrestha and Kim, 2007). Haemocytes are also able to phagocytose bacteria. It has been shown that *P. luminescens* secretes an effector called LopT (for luminescens outer protein T) through its type three secretion system (T3SS) (similar to YopT from *Y. pestis*) which supresses haemocyte's phagocytosis (Brugirard-Ricaud et al., 2005). Vlisidou and co-workers identified in 2010 a two-component regulatory system, KdpDE, based on the potassium concentration that allows *P. asymbiotica* to survive after phagocytosis within the insect's haemocytes. This study brought significant insights in the understanding of the survival of bacterial pathogen within phagocytic killing cells not only in insects but in mammals as well (Vlisidou et al., 2010) (see also Eleftherianos et al. 2010 for a review of *Photorhabdus* strategies to overcome the insect immune system). Besides the T3SS, *Photorhabdus* bacteria possess an alternative secretion system called *Photorhabdus* virulence cassette (PVC). PVC is a phage-tail-like structure which injects effectors that kill the haemocytes through dramatic actin cytoskeleton condensation (Yang et al., 2006). Another toxin produced by *Photorhabdus* species is the Mcf1 (for make caterpillars floppy) toxin, secreted by a dedicated Type 1 secretion system, which induces apoptosis in haemocytes or in a broad range of cultured human cells after internalisation (Daborn et al., 2002; Nick R Waterfield et al., 2009). No homolog of LopT has been found in *Xenorhabdus* which counteracts haemocyte phagocytosis thanks to enzymes (lipase, haemolysin, …) secreted through the flagella export apparatus (T3SS) (Park and Forst, 2006).

Entomopathogenicity of *Xenorhabdus* and *Photorhabdus* is also defined by their ability to effectively kill insects by producing insecticidal toxins. These toxins are highly pathogenic towards a wide range of insects. A dose as low as less than 5 *Xenorhabdus* or *Photorhabdus* CFU/insect is able to cause death within 48-72h after infection (Forst and Nealson, 1996; Milstead, 1979). Extensive studies have been conducted to identify the insecticidal toxin produce by *Xenorhabdus* and *Photorhabdus* bacteria in order to develop alternative strategies to control crop pest. Beside their entomopathogenicity, these toxins are able to inhibit phagocytosis helping bacteria to avoid the immune system (Lang et al., 2010).

The first insecticidal toxins were identified in *P. luminescens* as the "toxin complex" (Tc). In 1998, four distinct high molecular weight Tc were purified and named Tca, Tcb, Tcc and Tcd from *P. luminescens* and encoded by the loci termed with the same corresponding names. Both *tca* and *tcd* loci encode complexes orally toxic to *Manduca sexta*. Oral toxicity of *Photorhabdus* or *Xenorhabdus* secreted toxins greatly vary among bacterial strains and insect hosts. Indeed, cell-free supernatants from only 6 *Photorhabdus* strains out of 52 *Photorhabdus* and *Xenorhabdus* strains tested were orally toxic for thrips species (Gerritsen et al., 2005). These toxins revealed to be as toxic as *Bacillus thuringiensis* crystal toxin used to control crop pests in transgenic plants (Bowen et al., 1998). In *Photorhabdus*, the *tc* loci present two different types of organization. Both *tca* and *tcc* loci are organised in operons with three different open reading frames (ORF) transcribed in the same direction (*tcaA, tcaB, tcaC* and *tccA, tccB, tccC*). A shorter terminal ORF transcribed in the opposite direction is also present in *tca* and *tcc* loci (*tcaZ* and *tccZ*). The two last loci, *tcb* and its homolog *tcd* are both long ORF (*tcbA* and *tcdA*). However TcbA and TcdA are

cleaved in several (3 in the case of TcbA) polypeptides upon secretion. Figure 7 describes the genomic organisation of Tc ORFs in *Photorhabdus* and in *Xenorhabdus* and their respective homology (Ffrench-Constant and Bowen, 2000). The capital letters A to C determine the class of the toxin in the Tc, based on sequence similarity and size (Ffrench-Constant and Waterfield, 2006). Each Tc is composed of a combination of toxin proteins belonging to these 3 different classes. For example, the *Xenorhabdus* toxin complex 1 (XTc1) consists of three toxins XptA2, XptB1, and XptC1 with a stoichiometry 4:1:1 (Figure 6). The cytotoxic effect of the Tc is often linked to class A toxins (this is the case for XTc1)

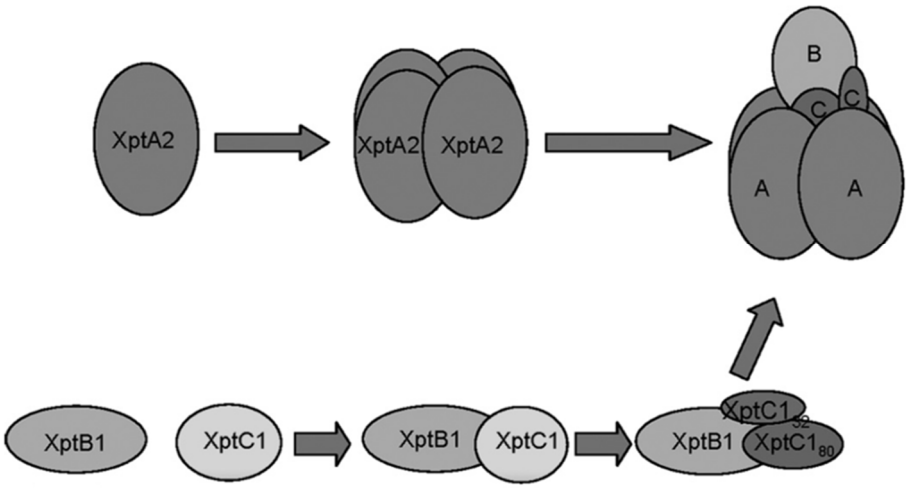

Figure 6: Putative model of complete native toxin complex 1 from *X. nematophilus*. The large XptA2 protein forms a homo-tetramer. The XptB1 and XptC1 proteins bind together to form a 1:1 dimer. The XptC1 protein is cleaved into a N-terminal fragment and a C-terminal fragment, both still bound to XptB1. The mechanism of cleavage is not known. This complex then binds to the tetrameric XptA2 to form the complete toxin complex. The exact location where XptB1 and XptC1 proteins bind to XptA2 is not known but is positioned on top of the complex for illustrative purposes (Adapted from Sheets et al., 2011).

and sometimes to class C toxins (for *Photorhabdus* toxin complex consisting of tccC3 and tccC5) whereas class B and C toxins modulate

and enhance the cytotoxic effect of the Tc (Guo et al., 1999; Lang et al., 2010; Sheets et al., 2011). *Photorhabdus* and *Xenorhabdus* Tc are so similar that XptA2 can interact with TcdB2 and TccC3 to form an active hybrid Tc that has greater insecticidal activity than the *Xenorhabdus* native Tc (Sheets et al., 2011). Tc-like genes have been found in other entomopathogenic bacteria like mammal pathogenic *Yersiniae* and *Serratia entomophila* (Waterfield et al., 2001). *Photorhabdus* possesses other insecticidal toxin genes encoding 2 Pir proteins, PirA and PirB (for *Photorhabdus* insect related). Pir proteins revealed to be orally toxic for mosquito larvae and lethal when injected to *G. mellonella* (Ahantarig et al., 2009; Waterfield et al., 2005). Another surprising toxin in *Xenorhabdus* is the HIP57 protein. This protein, which is highly toxic upon direct insect injection, is an homolog of the heat-shock chaperon protein GroEL (Yang et al., 2012). Two other *Xenorhabdus* toxins have been described in 2000 and 2004 (Brown et al., 2004; Keun et al., 2000)

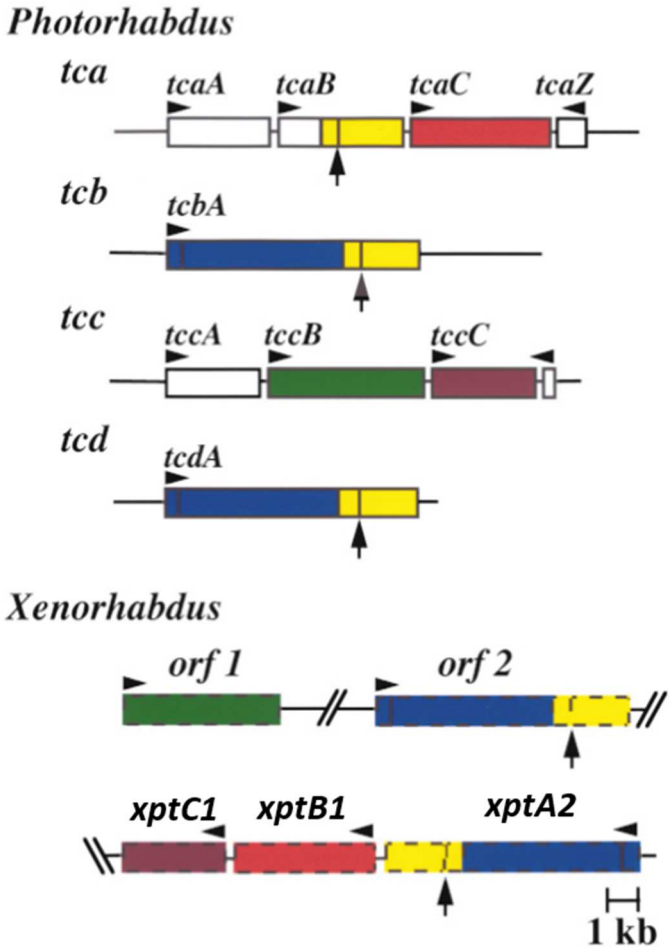

Figure 7: Genomic organisation of *Photorhabdus* and *Xenorhabdus* Tc ORF.

Both tca and tcc loci are organised in operons with three different open reading frames (ORF) transcribed in the same direction (*tcaA, tcaB, tcaC* and *tccA, tccB, tccC*). A shorter terminal ORF transcribed in the opposite direction is also present in *tca* and *tcc* loci (*tcaZ* and *tccZ*). The two last loci, *tcb* and its homolog *tcd* are both long ORF. Colors indicate homologs between *Photorhabdus* and *Xenorhabdus* Tc ORF. The clivage sites are showed by the vertical black arrows. (Adapted From Ffrench-Constant and Bowen, 2000)

3.4. *Xenorhabdus sp. and Photorhabdus sp. prevent microbial competition*

Besides killing the insect host and feeding the nematode, *Xenorhabdus* and *Photorhabdus* have to prevent microbial competitors' growth inside the insect's cadaver. A whole saprophytic population of bacteria and fungi are present in the insect waiting for an immunodeficiency of the insect host to develop. It has been shown that microbes appear in the hemolymph soon after insect invasion by EPNs (Gouge and Snyder, 2006). Paul and co-workers were the first, in 1981, to isolate and identify new anti-bacterial compounds produced by *Xenorhabdus* and *Photorhabdus* bacterial symbiont (Paul et al., 1981). From then, plenty of studies have been led to characterize the nature and the bioactivity of the products derived from both *Xenorhabdus* and *Photorhabdus* bacteria (Webster et al., 2002). There are two types of such products. The first one are large phage tail-like molecules called bacteriocins. These bacteriocins are bactericidal but display greater specificity towards bacteria closely related to EPNs symbionts or towards *Xenorhabdus* and *Photorhabdus* species or strains other than the symbiont producer. These bacteriocins clearly play a role in the species-specificity of nematode-bacteria symbiosis (Akhurst, 1982). The second type, only produced by form I variants, includes all other small non-proteinaceous secondary metabolites belonging to different chemical classes and displaying antibiotic, antimycotic, insecticidal or nematicidal activity. Both *Xenorhabdus* and *Photorhabdus* produce such secondary metabolites but *Xenorhabdus'* metabolites are more diverse than those from *Photorhabdus* (Webster et al., 2002). These metabolites not only have diverse chemical structures but also have a wide range of bioactivities with medicinal and agricultural interests such antiulcer, antineoplastic and antiviral. Methods are currently

developed to optimize the production of metabolites displaying interesting properties (Fang et al., 2012; Inman and Holmes, 2012; Inman III and Holmes, 2012; Y. Wang et al., 2011).

3.4.1. *Xenorhabdus* metabolites

A bacteriocin from *X. nematophila* was purified and named xenorhabdicin (Thaler et al., 1995). The phage tail-like xenorhabdicin is distinct from the phage particles present in the lysogenic strain of *X. nematophila*. It possess a high antibacterial activity against other *Xenorhabdus* species. Xenorhabdicins have thus a key role in preserving the specific symbiosis between *Steinernema* and *Xenorhabdus* (Thaler et al., 1995). Another enzyme produced by *Xenorhabdus* (and also by *Photorhabdus*) is chitinase. Chitinases inhibit the development of diverse fungi by destroying the fungal cell walls (Chen et al., 1994).

Besides bacteriocins and chitinases, *Xenorhabdus* bacteria produce small secondary non-proteinaceous metabolites derived from indoles (nematophin), dithiolopyrrolones (xenorhabdins and xenorxides) and xenocoumacins (close to amicoumacins from *Bacillus pumillus*). The indole derivatives are strongly active against Gram-positive and Gram-negative bacteria through RNA synthesis inhibition. Nematophins have demonstrated stronger antibacterial activity than the other indole derivatives (Li et al., 1997). Indoles have also shown some nematicidal effect. In vitro, indoles derivatives act as repellent for other *Steinernema* IJs (Hu et al., 1999). Xenorhabdins have a significant antibacterial effect on Gram-positive bacteria and yeast while almost no effect on Gram-negative bacteria. Some xenorhabdins exhibit insecticidal properties as well. (McInerney et al. 1991). Xenorxides possess the same pyrrolone nucleus but one of the two sulphate atom is oxidised. Despite the fact that they exhibit

comparable antimicrobial activity, the precise mode of action of these oxidised dithiolopyrrolones has not yet been reported (Webster et al., 2002). In general, dithiolopyrrolone compounds inhibit RNA and protein synthesis very quickly after exposure (20min) (Tipper, 1973) making them a precious arsenal during the early stage of insect infection. Xenocoumacins are highly active against Gram-positive bacteria like *Staphylococcus* and *Streptococcus* species and some Gram-negatives as well. Moreover, fungal species of *Aspergillus*, the yeast *Candida*, *Plasmodium* and *Trypanosoma* are also sensitive to xenocoumacins (McInerney et al., 1991b). In 2008, thanks to nuclear magnetic resonance (NMR), new metabolites were discovered and identified. One of them, termed xenematide, was active against some Gram-positive and Gram-negative bacteria but less efficient than xenocoumacins (Lang et al., 2008). The general structure of the compounds described before are presented in Figure 8. Research on new biologically active secondary metabolites, how to improve their production and pest control potential is still ongoing (Eom et al., 2014; Pidot et al., 2014; Schimming et al., 2014; Singh et al., 2014; Vizcaino et al., 2014). All the active metabolites are sumurized in the Table 1.

Table 1: Table describing the activity of the main active biological coumponds produced by *Photorhabdus* and *Xenorhabdus*

Xenorhabdus	*Photorhabdus*	Activity
Xenorhabdicin (Bacteriocin)	Photobactin / Luminicin (Bacteriocin)	Antibacterial towards closely related
Chitinase	Chitinase	Destruction of fungal cell wall
Nematophin (indol derivative)		RNA synthesis inhibition Gram+/- Nematicidal
Xenorhabdin / Xenorxide (dithiolopyrrolones)		RNA and protein inhibition in Gram+ and yeast Insecticidal
Xenocoumacine		Against Gram+/-, fungi and yeast
Xenematide		Gram+/-
	Anthraquinone	Bird repellent
	Hydroxystilbene	RNA synthesis inhibition Gram+/- Antifungal
	Photobactin	Antibacterial
	Carbapenem	β-lactamase antibiotics

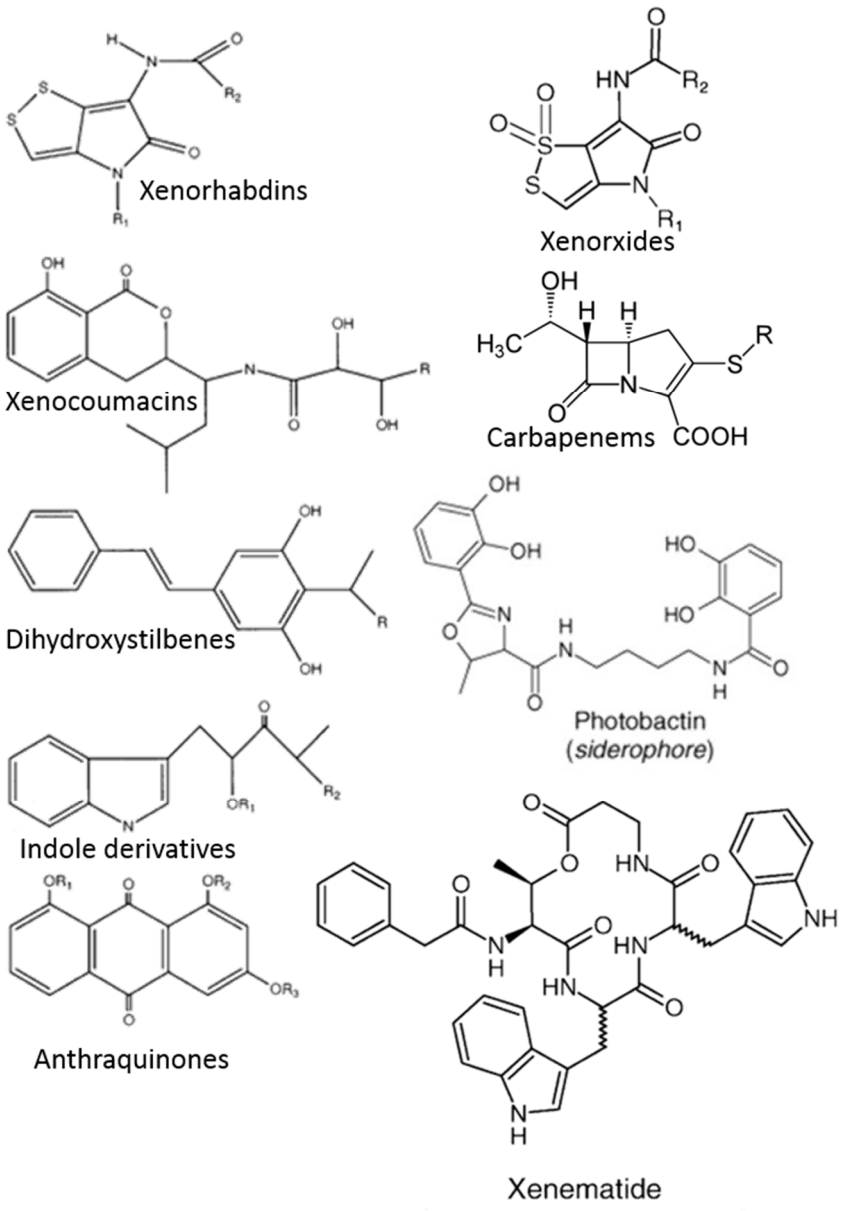

Figure 8: Biologically active secondary metabolites produced by *Xenorhabdus* and *Photorhabdus* bacteria.

Xenorhabdins and Photobactins are bacteriocins mainly active against closely related bacteria. Indole derivatives (like Nematophin) inhibit RNA synthesis of both gram positive and negative bacteria. They exhibit also a nematicidal activity. Xenorhabdins and Xenorxides are dithiolopyrrolone derivatives active against gram positive bacteria

and yeast. Xenocoumacines are active against a broad range of microbes like gram positive and negative bacteria, yeast and fungi. Xenematides produced by Xenorhabdus display antibacterial activities against gram positive and gram negative bacteria. Antibacterial activities of anthraquinones produce by *Photorhabdus* have not been demonstrated yet, but these pigmented molecules act as repellent for birds. Hydroxystilbenes are active against gram positive and negative bacteria by inhibing RNA synthesis but also act as antifungal compounds. Carbapenems are b-lactamase degrading the bacterial cell wall of both gram positive and negative bacteria.

3.4.2. *Photorhabdus* metabolites

Photorhabdus bacteria do release bacteriocins as well. One is called photorhabdicin, which is similar to the previously mentioned xenorhabdicin, and exhibit antibacterial activity against closely related bacteria (ffrench-Constant et al., 2003). Another one, called lumicin, is similar to colicins of *Escherichia coli*. Lumicins are able to break down genetic material from non-host cells thanks to their DNase and RNase activities (Sharma et al., 2002).

Hydroxystilbenes and anthraquinones are two chemical groups of non-proteinaceous secondary metabolites produced by *Photorhabdus*. Hydroxystilbenes share the same mode of antimicrobial action than indole derivatives by inhibiting RNA synthesis (Sundar and Chang, 1992; Webster et al., 2002). It is interesting to note that stilbenes are typical plant metabolites and until now, *Photorhabdus* is the only stilbene producer outside the plant kingdom (Bode, 2009). *Photorhabdus* stilbenes products are also involved as signal molecules in nematode development and counteract the insect immunity response. Anthraquinones might exhibit antibacterial and antimycotic activities (Li et al., 1995). However the major role of these pigmented molecules is to act as an ant and bird repellent to protect the infected insect from these predators (Pankewitz and Hilker, 2008). In 2002 and

2003, two other classes of molecules produced by *Photorhabdus* were described: carbapenems and siderophores. Carbapenems are a class of B-lactamase antibiotic while the *Photorhabdus* siderophore termed photobactin, possesses some antibiotic activities (Ciche et al., 2003; Derzelle et al., 2002). Moreover, several peptides including cytotoxic pentapeptides and yersiniabactin (another siderophore) derivatives could be identified in *P. asymbiotica* cultures (Nicholas R Waterfield et al., 2009). General chemical structures of *Photorhabdus* non-proteinaceous metabolites described above are presented in Figure 8.

4. Pathogenic Yersiniae

4.1. General Taxonomy and Phylogeny of Yersiniae

Bacteria of the genus *Yersinia* are facultative anaerobic psychrotrophic bacilli belonging to the family Enterobacteriaceae. Seventeen *Yersinia* species, discriminated from each other by significant DNA-based criteria and biochemical properties, have been identified so far (Savin et al., 2014). Among them, three are pathogenic to humans: *Yersinia pestis*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*. The two latter ones are qualified as enteropathogenic since they cause enteric disorders and cause infection through an oro-fecal mode of transmission (Carniel et al., 2002). *Y. pestis*, transmitted by rodent flea bites, is the etiological agent of plague (Perry and Fetherston, 1997; Stenseth et al., 2008). *Y. pestis* causes primarily the so-called bubonic form of plague after migrating from the insect bite to the proximal lymph node, resulting in the formation of a bubo. If not treated, the disease evolves to a systemic disease which is lethal in >90% of cases. Occasionally, the disease may evolve to a pneumonic stage resulting in massive invasion of the lungs by the plague bacilli.

Pneumonic plague can spread rapidly due to the highly contagious aerosols expectorated by infected individuals (Perry and Fetherston, 1997). *Yersinia ruckeri* is a fish pathogen causing the enteric redmouth disease in salmonid fish like the rainbow trout (Ewing et al., 1978). *Yersinia similis*, termed that way for its close genetic proximity with *Y. pseudotuberculosis*, has been isolated from rabbit and surface water but has never been isolated from diseased or dead mammals (Sprague et al., 2008). Another *Yersinia* was identified as a member of the *Y. pseudotuberculosis* group. This species was isolated in Korea and has been differentiated from *Y. pseudotuberculosis* thanks to Multiple Locus Sequence Typing (MLST), a molecular typing technique based on the nucleotide sequencing of selected house-keeping genes (Laukkanen-Ninios et al., 2011a), and was named *Yersinia wautersii*. Conversely to *Y. similis*, Y. *wautersii* can cause human and animal gastro-enteric infection (Savin et al., 2014). Comparatively, other *Yersinia* species are closely related to *Y. enterocolitica*: *Yersinia krirtensenii*, *Yersinia intermedia*, *Yersinia mollaretii*, *Yersinia frederiksenii* and *Yersinia bercovieri*. No human or animal pathogenic potential has been reported for these *Y. enterocolitica*-like species. Other *Yersiniae* diverged into non-pathogenic species: *Yersinia aldovae*, *Y. aleksiciae*, *Y. rohdei* and *Y. massiliensis* (Sulakvelidze 2000; Sprague and Neubauer 2005; Merhej et al. 2008). In 2011, a novel species of *Yersinia* was isolated from a diseased larva of a Scarabaeidae from New Zealand. This *Yersinia* species was shown to be entomopathogenic towards a broad range of insects and was thus termed *Yersinia entomophaga* (Hurst et al. 2011; Hurst et al. 2011). Even if *Y. wautersii*, *Y. ruckeri* and *Y. entomophaga* are animal pathogens, in this work we will gather under the name "pathogenic *Yersiniae*" the three following species: *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*.

Although they cause the same disease and have the same transmission mode, *Y. enterocolitica* and *Y. pseudotuberculosis* diverged from a distant common ancestor (41 – 186 million years ago). Despite its totally different etiology and mode of transmission, *Y. pestis* evolved from *Y. pseudotuberculosis* very recently (no more than 20,000 years ago). Indeed, while *Y. pseudotuberculosis* and *Y. enterocolitica* are food- and water-borne pathogens usually transmitted by the faecal-oral route, *Y. pestis* uses fleas as vector to infect new hosts (mainly rodents) (Achtman et al., 1999; Wren, 2003). The main difference between Y. *pseudotuberculosis* and *Y. pestis* is the acquisition by the latter of extra virulence plasmids (Table 2). The three pathogenic *Yersiniae* do possess one large highly-conserved plasmid (about 70kb) called pYV (for *Yersinia* Virulence) or pCD (for Calcium Dependence) which encodes virulence factors such as the *Yersinia* adhesine (YadA) or Outer membrane Proteins (Yops), which are virulent effectors secreted by a Type Three Secretion System composed of the Ysc proteins (Yop Secretion) and encoded by the same plasmid. In vitro, the presence of this plasmid confers a so-called low-calcium response (Lcr) phenotype rendering bacteria unable to grow at 37°C in the absence of Ca2+. This is an ecological regulation mechanism avoiding virulence factors production outside a susceptible mammal host (Straley and Bowmer 1986; Cornelis et al. 1998; Zadernowska et al. 2013). In addition to this common plasmid, *Y. pestis* acquired two other plasmids termed pFra (or pMT for murine toxin) and pPla. The former encodes a phospholipase D, described as a murine toxin (Ymt) required for the colonization of the plague flea vector (Hinnebusch et al., 2002). The pPla (for Plasminogen activator) plasmid encodes a protease termed Pla that activates plasminogen in order to facilitate circulation of the plague bacillus in the infected blood (Lathem et al., 2007). Together

with a high pathogenicity island (HPI) and a number of minor genetic mutations in the *Y. pestis* genome as compared to *Y. pseudotuberculosis*, these plasmids cause the deadly famous disease *Y. pestis* is sadly known for.

Table 2: Virulence plasmids repartition through human pathogenic *Yersiniae*

Plasmid name	Host species	Main virulence factors encoded
pYV (or pCD)	*Y. enterocolitica* *Y. pseudotuberculosis* *Y. pestis*	Adhesines Yops T3SS
pMT (or pFra)	*Y. pestis*	Murine toxin (phospholipase D)
pPla	*Y. pestis*	Plasminogen activator protease

There is a huge diversity among enteropathogenic *Yersiniae* while *Y. pestis* is a monomorphic clone of its parental species, *Y. pseudotuberculosis*. *Y. enterocolitica* is subdivided into 6 biotypes based on their biochemical characteristics and pathogenicity (1A, 1B, 2, 3, 4 and 5). Biotype 1B is thought to be the most pathogenic to human (Zadernowska et al. 2013). *Y. enterocolitica* can also be subdivided into 76 distinct serotypes based of the variable structure of the O-specific polysaccharide chain of the LPS. Only a few serotypes are pathogenic. O:3 is found primarily in Europe, Canada, China and Australia, O:8 is mainly found in the USA and Japan and O:9 causes gastro-intestinal infections in Scandinavia, the Netherlands and China (Sabina et al., 2011). There are 15 distinct major O-serotypes among *Y. pseudotuberculosis* which are subdivided into 6 genogroups according

to their virulence factors (plasmid and HPI) content (Laukkanen-Ninios et al., 2011b). Like for *Y. enterocolitica*, the prevalence of *Y. pseudotuberculosis* serotypes varies geographically. In Europe, the most encountered serotypes are O:1, O:2 and O:3 while in Japan isolates most often belong to serotypes O:4b, O:3, O:5a, and O:5b. *Y. pestis* cannot be divided into serotypes because it does not express the LPS O-specific polysaccharide chain (Castro et al., 2009). Based on some minor biochemical properties, *Y. pestis* has been subdivided into 4 biovars: Antiqua (from the Justinian plague around the Mediterranean basin during Antiquity period), Medievalis (from the Black Death and subsequent epidemics in Europe between 1346 and the early 19th century), Orientalis (from China ad spread globally via marine shipping from 1860s till now) and Pestoides (from Russian and Mongolian enzootic isolates) (Anisimov et al., 2004; Morelli et al., 2010; Perry and Fetherston, 1997). If variations in biochemical activities exist between these 4 biovars, it is unlikely that each of them are specifically related to the time of the plague epidemic waves their names refer to. This was demonstrated after the finding of *Y. pestis* Orientalis DNA from European burial places aged from the 12th – 15th centuries (Haensch et al., 2010; Tran et al., 2011; Welford and Bossak, 2010).

4.2. Persistence of pathogenic Yersiniae in the environment

In spite of the fact that these are non-spore-forming bacteria, *Yersiniae* can survive for long periods in the environment. This is particularly true for plague which can disappear and re-emerge after years or decades exactly at the same places (Bertherat et al., 2007). Currently, *Yersinia* persistence in the environment remains just partially or not at all elucidated. From soil to resistant and/or

asymptomatic hosts, many hypothesis and models have been proposed to explain the ability of these enzootic agents to persist in the environment and repeatedly cause outbreaks and epidemics. We will describe hereafter the natural hosts of the three pathogenic *Yersiniae* and their ability to persist among their host population. Then we will describe the reported maintenance of these *Yersiniae* in soil.

4.2.1. Natural hosts

4.2.1.1. Yersinia enterocolitica

The main reservoir for *Y. enterocolitica* are pigs which are also an important source of *Y. enterocolitica* human infection. *Y. enterocolitica* is also present in other animals like cats and dogs, poultry and sheep, rodent, birds and wild boars. After excretion from the host gut, *Y. enterocolitica* can survive a long time in the environment due to its resistance to unfavourable conditions (Zadernowska et al. 2013). It has been reported that *Y. enterocolitica* can be present on vegetables as well and cause human infection when raw material are consumed. These vegetables can be contaminated by organic fertilizer or during their processing and packaging. As a psychrotrophic bacteria, a strict respect of the cold chain will not prevent *Y. enterocolitica* from growth in contaminated food (Beuchat, 2002).

4.2.1.2. Yersinia pseudotuberculosis

Y. pseudotuberculosis mainly infects a broad range of both sylvatic and domestic mammals and birds similarly to *Y. enterocolitica* (Fukushima et al., 1989). Human infections, though less common than with *Y. enterocolitica*, occur via ingestion of contaminated food or water. Pets (cats and dogs) are an important source of human

contaminations (Fukushima et al., 1985). Additionally, *Y. pseudotuberculosis* has been found in vegetables (carrot and salad) and led to children infection (Jalava et al., 2006).

4.2.1.3. Yersinia pestis

Y. pestis mainly infects rodents while humans are only accidental hosts. Conversely to its parental *Y. pseudotuberculosis*, *Y. pestis* is not transmitted by the faecal-oral route. Rather, *Y. pestis* uses fleas as vector for transmission from a rodent to another. Fleas become infected when fed on contaminated blood. Upon infection, *Y. pestis* bacteria colonize the upper part of the gastro-intestinal tract (more precisely the proventriculus) of the flea through biofilm formation. Bacteria multiplies and form cohesive aggregates to eventually block normal blood feeding. It has been shown that the so-called 'murin toxin' Ymt is required for *Y. pestis* to colonize flea's midgut and proventriculus (connection between oesophagus and midgut). Infected fleas unable to feed bite actively and randomly (including non-specific host) led by their starvation. During their persistent efforts to feed, plague bacilli are dislodged from the proventriculus aggregates into the bite site infecting a new host. Prevented to feed, some of the infected fleas will eventually die from starvation but some of them will survive after autolysis of the *Y. pestis* clots in the proventriculus (Bacot and Martin, 1914; Chouikha and Hinnebusch, 2012; Hinnebusch et al., 2002). *Y. pestis* life cycle is primarily sylvatic among a variety of wild rodents like field rats, prairie dogs or squirrels and their specific fleas. Sometimes, domestic rodents (commensal rats) or even pets (cats and dogs) can be infected with *Y. pestis* bacteria and carry infected fleas, increasing at the same time the probability of human infection (Gasper et al., 1993; Gould et al., 2008). Mammal to

mammal transmission of the plague bacillus is non-common but still possible since *Y. pestis* can infect some mammal by the oral route (for example carnivorous mamals feeding on a infected cadaver) (Arbaji et al., 2005; Gasper et al., 1993) or be propagated via aerosol droplets in the plague pulmonary stage (Perry and Fetherston, 1997).

4.2.2. Soil persistence

4.2.2.1. Yersinia enterocolitica

It has been shown that the survival of non-pathogenic serotypes of *Y. enterocolitica* in soil and water is longer than survival of pathogenic serotypes in vitro (Tashiro et al., 1991). However, pathogenic *Y. enterocolitica* are detected in the soil of infected animal farms (Botzler, 1979; Hughes, 1979). Besides its ability to survive in the environment, *Y. enterocolitica* might interact with free-living organisms or use them as a biological reservoir. Supporting this hypothesis, Lambrecht and co-workers recently showed enhanced survival of *Y. enterocolitica* in co-cultivation with free-living amoeba *Acanthamoeba castellanii*. Thanks to TEM analysis, they localized *Y. enterocolitica* in the cytosol of *A. castellanii* avoiding the digestion in the food vacuole of the amoeba. They also showed that uptake by the amoeba of virulent strains is higher than the uptake of non-virulent ones (Lambrecht et al., 2013).

4.2.2.2. Yersinia pseudotuberculosis

Soil and water persistence abilities of *Y. pseudotuberculosis* have also been demonstrated (Buzoleva and Somov, 2003). Zureck and co-workers recovered *Y. pseudotuberculosis* from the intestinal tract of house fly larvae (*Musca domestica*) and demonstrated that adult house

flies can carry *Y. pseudotuberculosis* for a long time. This insect vector may favour the spreading and the maintenance of *Y. pseudotuberculosis* in the environment (Rahuma et al., 2005; Zurek et al., 2001, 2000).

4.2.2.3. Yersinia pestis

As the other pathogenic *Yersiniae*, *Y. pestis* is able to persist in the soil and in water (Ayyadurai et al., 2008; Eisen et al., 2008; Gilbert and Rose, 2012). However, *Y. pestis* has established several permanent enzootic foci which are geographically not connected resulting in considerable ecological disparities (Anisimov et al. 2004). The maintenance of *Y. pestis* in nature depends on a complex ecology involving many different rodents and their associated fleas. It appears then that unlike the other pathogenic *Yersiniae*, environmental survival is no longer needed for *Y. pestis* to maintain but instead the balance between to eukaryotic hosts, insects and mammals, plays a more important role in its maintenance (Chouikha and Hinnebusch, 2012). Even if naturally resistant rodents are well-known to act as biological reservoir in enzootic foci, the causes of cyclic reappearance of plague outbreaks in some areas are still poorly understood (Arbaji et al., 2005; Bertherat et al., 2007). It has been observed that climatic condition and regional topography have an influence on the timing of human outbreaks and epidemics. These factors directly impact the behaviour of rodents and fleas. Combined with the local human behaviour and organisation, they might explain the maintenance of plague foci. All reviews and studies led until now to the conclusion of *Y. pestis* maintenance in particular and distinctive worldwide foci, while revealing the extreme complexity and specificity of plague foci (Andrianaivoarimanana et al., 2013; Ben Ari et al., 2011; Brouat et al.,

2013; Eisen and Gage, 2009; Eisen et al., 2012; Eorge et al., 2013; Gascuel et al., 2013; Hubbart et al., 2011; Laudisoit et al., 2009; Stapp et al., 2004).

4.3. Entomopathogenicity of Yersiniae

The three mammal pathogenic *Yersiniae* display some pathogenicity toward insects as well. In particular, *Yersiniae* do possess homologs of the Tc insecticidal complex of *Photorhabdus* (Waterfield et al., 2007). Moreover, it has been suggested that *Y. pestis* acquired its plasmid-encoded *ymt*, required for flea colonization, from *P. luminescens* or from a close relative (Duchaud et al., 2003). In addition, *P. asymbiotica* which can infect either insects or humans, possesses a plasmid related to pMT-1 found in *Y. pestis* (Wilkinson et al., 2009). All these homologies between pathogenic *Yersiniae* and *Photorhabdus* pointed insects as a potential biological reservoir for *Yersiniae* (Fuchs et al., 2008). Altogether, this could explain the well-known entomopathogenicity even if it varies among pathogenic *Yersiniae* species. Hereafter, we will compare the entomopathogenicity of pathogenic *Yersiniae* and make the difference here between two major components of entomopathogenicity which are the ability to colonize insects on one hand and the ability to effectively cause insect's death through insecticidal toxin release on the other hand.

4.3.1. *Yersinia enterocolitica*

Y. enterocolitica is entomopathogenic since it can not only colonize insects' body and cells but also uses a panel of insecticidal toxins to kill the insect host. Heermann and Fuch made a genome comparison between *Y. enterocolitica* and *P. luminescens*. Among other genes they showed that the insecticidal Tc complex is present in both

bacteria confirming the work of Waterfield and co-workers (Waterfield et al. 2001; Heermann and Fuchs 2008). The insecticidal activity of *Y. enterocolitica* is induced under low-temperature conditions (10°C) while it is repressed at temperatures above 30°C. In its natural environment (Northern hemisphere), where the temperatures are most of the time below 30°C, *Y. enterocolitica* is thus able to invade and kill insect larvae such as *G. mellonella* and *M. sexta*. This points out the dual pathogen behavior of *Y. enterocolitica*, towards both insects and mammals (Fuchs et al., 2008).

4.3.2. *Yersinia pseudotuberculosis*

Y. pseudotuberculosis exhibit some insecticidal activities as well. For example, it is able to infect and kill *G. mellonella* larvae (Champion et al., 2009). However, unlike in *Y. enterocolitica*, Tc genes are expressed either at 15°C or 30°C. In addition, *Y. pseudotuberculosis* exhibit weak oral toxicity towards *M. sexta* while heterologous expression in *E. coli* of the *Y. pseudotuberculosis tca* genes effectively kills *M. sexta* (Fuchs et al., 2008; Pinheiro and Ellar, 2007). *Y. pseudotuberculosis* insecticidal *tc* genes are not involved in virulence against flea while the *Photorhabdus* Tc products are lethal for the same flea (Erickson et al., 2007). Interestingly, *Y. pseudotuberculosis* shows acute oral toxicity against *X. cheopis* fleas. Without killing them, *Y. pseudotuberculosis* is able to colonize the house fly's gut and use it as a mechanical vector (Zurek et al., 2001).

4.3.3. *Yersinia pestis*

Among the three pathogenic *Yersiniae*, *Y. pestis* is the less entomopathogenic. This is quite understandable since *Y. pestis* uses fleas as vector. As mentioned before *Y. pestis* colonizes the flea's

midgut preventing them to feed. If some of the infected fleas will effectively die from starvation, the others will survive after autolysis of the *Yersinia* clots. *Y. pestis* also possesses homologs of *Photorhabdus* Tc genes, like *tca* and *tcc*. While Tc products from *P. luminescens* demonstrated acute oral toxicity against *M. sexta* larvae, *Y. pestis* homologs had no effect neither on *M. sexta* nor on the rat flea *X. cheopis*. If they display poor insecticidal activity, the *tca* operons of *Y. pestis* and *Y. pseudotuberculosis* seem adapted to mammalian hosts. Modification of the gut epithelium caused by *Y. pseudotuberculosis* and involvement in the initial invasion of the mammalian host after transmission by fleas in the case of *Y. pestis* are supportive to this statement. Moreover it appears that a similar adaptation also happened to the *tca* operon of *P. asymbiotica* allowing it to infect insects and mammal hosts (Waterfield et al., 2007).

Objectives

Persistence of pathogenic bacteria in the environment remains an international health issue. The existence of biological micro-reservoir ensuring the maintenance of such pathogens in a given environment has been postulated, but this hypothesis remains misunderstood especially regarding pathogenic *Yersinia*. Soil invertebrates have been particularly suspected to act as intermediary hosts, thus in this study, entomopathogenic nematodes (EPNs) will be investigated in this respect. Based on their phylogenetic proximity to *Xenorhabdus* and *Photorhabdus* bacteria, a model assuming *Yersinia's* ability to colonize the same ecological niche as these two nematode symbionts will be set up. The main objective of this work is to evaluate the ability of human pathogenic *Yersinia* to colonise EPNs in a laboratory model. This model consists in insect larvae, *Steinernema* EPNs with or without their natural *Xenorhabdus* symbiont and *Yersinia* brought artificially either in the gut of EPNs or in the haemocoele of the insect larva prior to infection.

The first step will consist in checking if *Yersinia* are able to colonize *Steinernema* EPNs using confocal microscopy and fluorescent-labelled bacteria. The resistance of *Yersinia* towards *Xenorhabdus* antimicrobials will be tested as well as the long-term persistence of *Yersinia* through EPNs infection cycles. Other enterobacteria will also be tested in order to challenge the specificity of the *Steinernema* colonization by *Yersinia*. Second, genetic determinants potentially involved in the colonization of EPNs will be deleted. The resulting knockout mutants will be complemented and the phenotypes will be studied using the same laboratory model. This work will bring new insights in the underestimate role of microinvertebrate in the pathogenic bacterial persistence in the environment.

Chapter 1 – Method for fluorescent marker swapping and its application in *Steinernema* nematode colonization studies.

This work has been published in Journal of Microbiological Methods[1] and adapted for this thesis purpose.

[1] Gengler S, Batoko H, Wattiau P (2015) Method for fluorescent marker swapping and its application in *Steinernema* nematode colonization studies. Journal of Microbiological Methods, vol. 113, pp 34-37.

Chapter 1 – Method for fluorescent marker swapping and its application in *Steinernema* nematode colonization studies.

Abstract

An allelic exchange vector was constructed to replace *gfp* by *mCherry* in bacteria previously tagged with mini-Tn*5* derivatives. The method was successfully applied to a *gfp*-labeled *Yersinia pseudotuberculosis* strain and the re-engineered bacterium was used to study the colonization of *Steinernema* nematodes hosting their *Xenorhabdus* symbiont using dual-color confocal microscopy.

Fluorescent markers are largely used to quantify or to track microorganisms in various microbiological studies. The most common labeling system is based on the Green Fluorescent Protein (GFP) originally isolated from *Aequorea victoria* (Heim et al., 1994). Genetic tagging supposes that the *gfp* gene is brought inside the target organism either via a mobile episome (plasmid, transposon) or by site-specific recombination at a pre-identified genetic locus. When dealing with gram-negative bacteria with limited genetic engineering possibilities, mini-transposons are valuable options to consider for conducting strain tagging. Mini-transposons are modified versions of original elements bearing an antibiotic resistance marker, an optional marker typically conferring fluorescence, luminescence or an easily detectable enzymatic activity and devoid of transposase, the latter being brought transiently together with the mini-transposon delivery vector in the target strain. The mini-Tn-*gfp* family, initially developed in 1996 (Christensen et al., 1996) and further improved in several subsequent works (Eberl et al., 2006; Errampalli et al., 1999; Matthysse and Dandie, 1996) was successfully used in a variety of microbiological studies ranging from simple biofilm formation to sophisticated pathogenicity model experimentations (Cho and Kim, 1999; Gjermansen et al., 2005; Richter and Smalla, 2007; So et al., 2002; A. Wang et al., 2011).

When applied to complex biological systems, the success of GFP-labeling is however mitigated because of interferences frequently occurring between the fluorescent marker and components of the environmental matrix. Bleaching and quenching are the most common of such interferences (McVey and Crain, 2014) and the choice of the fluorescent label is therefore critical. In a similar way, co-localization studies requiring different markers rely on carefully selected

fluorescent labels displaying non-interfering fluorescence characteristics. The choice of available fluorescent markers was limited in the early times of fluorescent organism tagging but rapidly improved following the discovery of new fluorescent proteins and the engineering of GFP variants displaying brighter fluorescence, shifted light excitation and/or emission spectra and improved robustness (Ai et al., 2007; Cubitt et al., 1995; Shaner et al., 2005). Nowadays, while the number of available fluorescence markers has exploded (Shaner et al., 2005), the palette of mini-transposons available for strain tagging is still limited. Considering the work required to obtain transposon-labelled bacterial strains having the desired genetic and physiological characteristics with the right fluorescence expression level, systems allowing fluorescent marker swapping in previously characterized mini-transposon insertion mutants would allow re-engineering of such mutants with new label characteristics.

The aim of the present work was to develop an allelic exchange vector able to replace *gfp* or variants thereof by *mCherry*, a gene coding for a red fluorescence protein with spectral characteristics optimized for double labelling with GFP (Shaner et al., 2005). A fluorescence exchange cassette was generated using an overlap PCR protocol adapted for short fragments as described by Shevchuck et al. (Shevchuk et al., 2004). Three PCR fragments were amplified, a central fragment (CF) and two flanking fragments located upstream and downstream (UF and DF, respectively) as described in Figure 9. CF contains *mCherry* nucleotide sequence with a ribosome binding site added upstream to ensure optimal translation. UF and DF are used for homologous recombination and target the *gfp* gene already present in the mini-transposon.

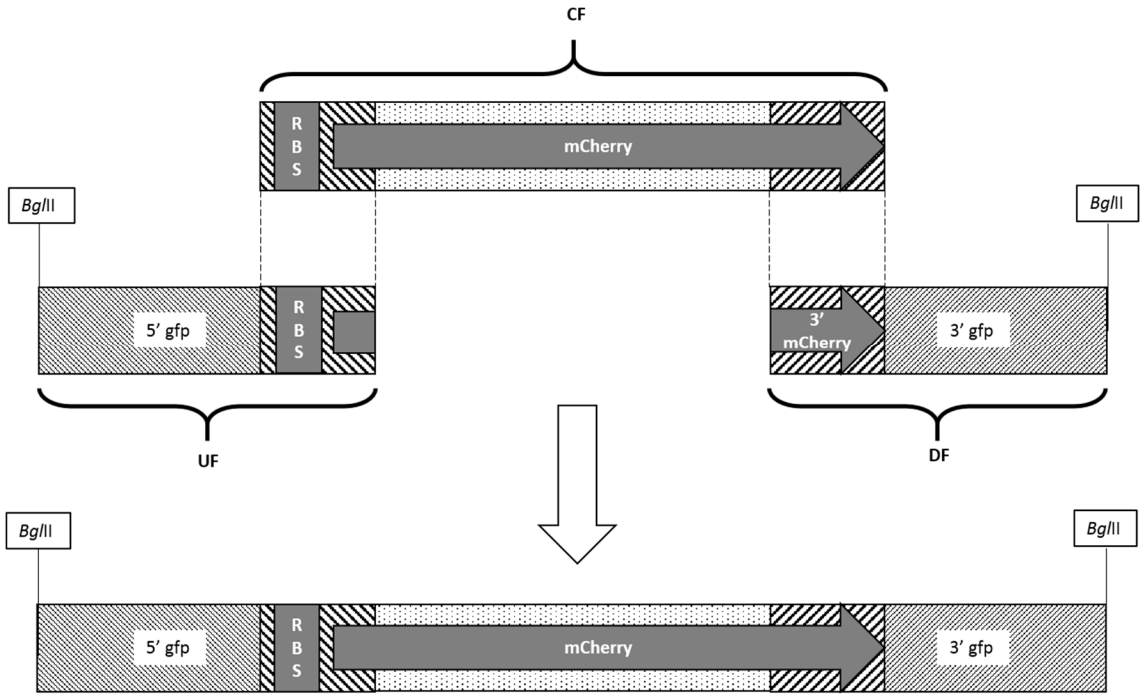

Figure 9: Schematic view of the fluorescence exchange cassette.

UF and DF consist of 244 and 223 bp from the *gfp* coding sequence, respectively. CF consists of the *mCherry* coding sequence with a RBS added upstream. Overlapping sequences are shown in spaced hatched bars. The whole cassette is generated by a short triple fusion PCR (Shevchuck et al., 2004). The cassette is flanked by *Bgl*II restriction sites.

Table 3: List of Primers used in this study

Name	Sequence (5′ to 3′)	Description	Amplicon[1]
BG883	CCGAGATCTGCC*TGGAGTTGTCCCAATTC TTGTTGA*	*gfp* forward primer with *Bgl*II restriction site (underlined) and nucleotide sequence matching bases 27 to 50 of *gfp*[2] (italics)	UF
BG884	*TTGCTCAACAT*TCCGATTTCCTCCTTGGT CACTAGTTTCGGGCATGGCACTCTT	*gfp* reverse primer with nucleotide sequence matching bases 270 to 253 of *gfp*[2] (underlined) and bases 11 to 1 of *mCherry*[3] (italics)	
BG885	*GCTGTACAAGTAA*GCAGGCGCAAAATTA GACACAACATTGAAGATGGAAGC	*gfp* forward primer with nucleotide sequence matching bases 495 to 525 of *gfp*[2] (underlined) and bases 699 to 711 of *mCherry*[3] (italics)	DF
BG886	GGCAGATCTCGGTTA*TTTGTATAGTTCAT CCATGCCATGTGTAATC*	*gfp* reverse primer with *Bgl*II restriction site (underlined) and nucleotide sequence matching bases 714 to 684 of *gfp*[2] (italics)	
BG887	*GTGACCAAGGAGGAAATCGGAATGTTGA GCAA*GGGCGAGGAGG	*mCherry* forward primer with ribosome binding site and start codon (underlined), overlap sequence with UF (italics)	CF
BG888	*CAATGTTGTGTCTAATTTTGCGCCTGCTTA CTTGTACAGC*TCGTCCATGCC	*mCherry* reverse primer with stop codon (underlined), overlap sequence with DF (italics)	

[1] As specified in Fig.1. UF, CF and DF: Upstream, Central and Downstream PCR Fragments

[2] GenBank Accession Number KC551806

[3] GenBank Accession Number AY678264.1

The primers used are listed in Table 3. UF, DF and CF were separately amplified using 30 PCR cycles each consisting in 30 s at 95°C, 30 s at 60°C and 1min at 72°C followed by a final elongation at 72°C for 10min. The full-length exchange cassette was generated by triple fusion PCR using primers BG883 and BG886 as described (Shevchuk et al., 2004). The final PCR product was restricted by *Bgl*II and ligated into the unique compatible *Bam*HI site of the mobilizable suicide vector pKNG101, which confers resistance to streptomycin and carries the counter-selectable marker *sacBR* (the bacteria is unable to grow when sucrose is added in the medium) (Kaniga et al., 1991). After *Bam*HI re-digestion of the ligation product to eliminate the self-ligated plasmids, the resulting plasmid termed pSGCG (= LMBP 9561) was electroporated in *E. coli* SM10 λPir resulting in *E.coli* 10WP. To assess the functionality of the developed vector, pSGCG was conjugated into *Y. pseudotuberculosis* 4N1G, a nalidixic-acid resistant field isolate labelled with a mini-Tn*5-gfp* transposon (Gengler et al., 2015b). Allelic exchange was conducted in two steps. Initial integration of pSGCG was first selected on specific agar plates containing nalidixic-acid (35µg ml^{-1}) and streptomycin (100µg ml^{-1}). After purification of the recombinant strain, a second recombination event was selected on agar plates containing nalidixic-acid and sucrose (100µg ml^{-1}). Recombinant *Y. pseudotuberculosis* 4N1C colonies expressing mCherry but not GFP were validated by both epifluorescence microscopy and PCR. To evaluate the suitability of combined mCherry and GFP fluorescent proteins in dual-labeling experiments, *Steinernema* entomopathogenic nematodes (EPNs) hosting a GFP-tagged derivative of their natural symbiont *Xenorhabdus* sp. TZ03 (Gengler et al., 2015b) were allowed to graze on a Wouts agar plate covered with a lawn of the mCherry-tagged *Y. pseudotuberculosis* 4N1C described above. After one week,

EPNs harboring the two fluorescent bacteria were stored for 48h at 4°C in physiological water, a treatment that kills the nematodes while allowing *Xenorhabdus* and *Yersinia* to survive and slowly multiply. Confocal imaging was then conducted using a Zeiss LSM710 confocal microscope equipped with a spectral detector and a water-immersion x 40 objective. GFPmut2 and mCherry proteins were imaged simultaneously using a 488-nm excitation laser for GFPmut2 and a 561-nm excitation laser for mCherry. Light was amplified and recorded through 505 to 530 nm (GFPmut2) and 595 to 640 nm (mCherry) filters. Confocal images were processed using the ZEN2012 blue edition software (Zeiss, Oberkochen, Germany). While *Xenorhabdus* sp. TZ03 clustered in the so-called symbiotic vesicle of the nematodes, *Y. pseudotuberculosis* 4N1C colonized the intestinal tract (Figure 10A). Thus, the presence of *Y. pseudotuberculosis* does not affect the vesicle localization of *Xenorhabdus*. The simultaneous EPNs colonization of *Xenorhabdus* and *Y. pseudotuberculosis* is also observed in the EPN's male and female adult stages (distinguishable from IJs by the presence of sexual organs) (Figure 10B and C). In some EPN males only, colonized or not by *Y. pseudotuberculosis* 4N1C, we observed a break-up of the symbiotic vesicle which has never been reported before (Figure 10B, D and E). This may be the hallmark of an intermediate stage during the juveniles' maturation into male adults.

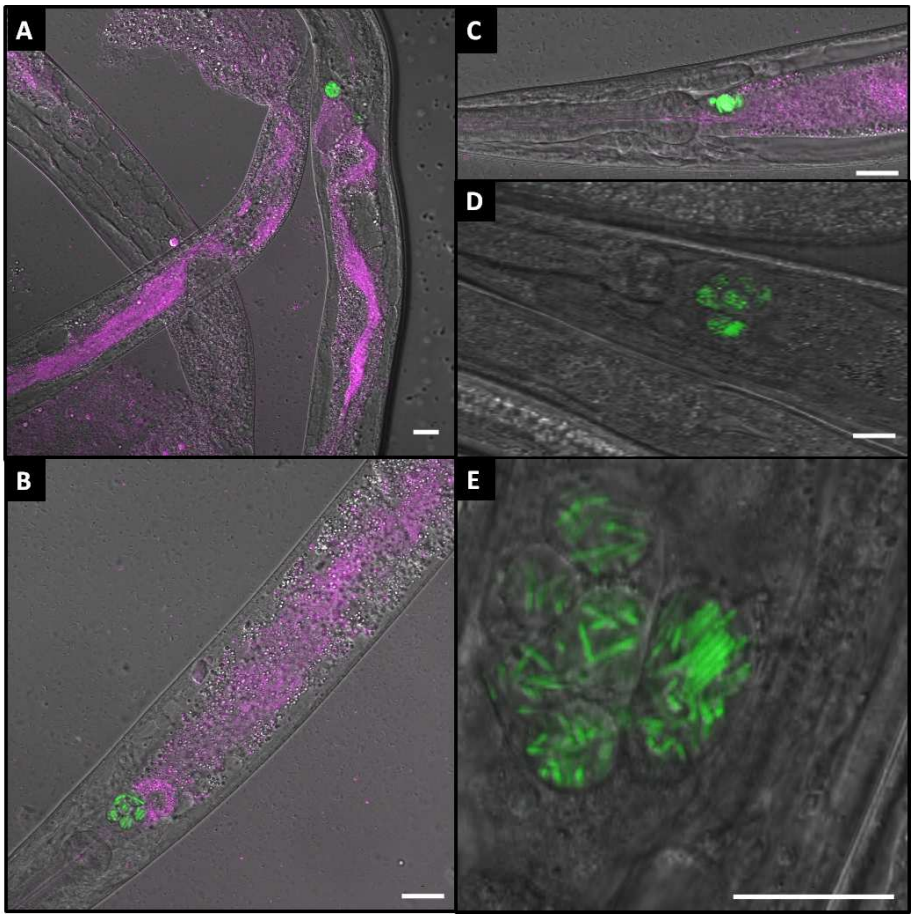

Figure 10: Confocal microscope images of GFP-labelled *Xenorhabdus* TZ03 and mCherry-labelled *Y. pseudotuberculosis* 4N1C in *Steinernema*. sp. MW8B EPNs. White scale bars = 20µm.

A. *Steinernema* IJ simultaneously colonized by *Xenorhabdus* TZ03 (confined in the symbiotic vesicle) and *Y. pseudotuberculosis* 4N1C (scattered along the gut).
B. *Steinernema* male simultaneously colonized by *Xenorhabdus* TZ03 and *Y. pseudotuberculosis* 4N1C.
C. Young *Steinernema* female simultaneously colonized by *Xenorhabdus* TZ03 and *Y. pseudotuberculosis* 4N1C.
D. *Steinernema* male colonized only by *Xenorhabdus* TZ03 (confined in split vesicles).
E. Enlarged view of the split vesicles shown in D.

In conclusion, the fluorescence replacement vector described herein was successfully assessed and validated on a mini-Tn*5*-*gfp* tagged *Y. pseudotuberculosis* strain, resulting in a *mCherry*-labeled derivative with genetic characteristics identical to the parent strain. The re-engineered strain was used for in vivo colonization studies of *Steinernema* entomopathogenic nematodes hosting their natural *Xenorhabdus* bacterial symbiont tagged with GFP. Both GFP- and mCherry-labeled fluorescent bacteria could be readily differentiated by confocal microscopy. Due to the high nucleotide sequence similarity of most of the *gfp* variants engineered so far, *gfp* to *mCherry* allelic exchange catalyzed by pSGCG is likely to work in many other situations where a gram-negative bacterium tagged with *gfp* or one of its variants needs to be re-engineered.

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Chapter 2 - Long-term persistence of *Yersinia pseudotuberculosis* in entomopathogenic nematodes.

This work has been published in Plos ONE[1] and adapted for this these purpose.

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Chapter 2 - Long-term persistence of *Yersinia pseudotuberculosis* in entomopathogenic nematodes.

Abstract

The existence of biological micro-reservoirs for pathogenic bacteria explaining the long-term survival of these organisms in the environment has long been speculated. Soil invertebrates have been particularly suspected to act as intermediary hosts and entomopathogenic nematodes (EPNs) were here investigated in this respect. EPNs are small worms whose ecological behaviour consists to invade, kill insects and feed on their cadavers thanks to a species-specific symbiotic bacterium belonging to any of the genera *Xenorhabdus* or *Photorhabdus* hosted in the gastro-intestinal tract of EPNs. The symbiont provides a number of biological functions that are essential for its EPN host including the production of entomotoxins, of enzymes able to degrade the insect constitutive macromolecules and of antimicrobial compounds able to prevent the growth of competitors in the insect cadaver.

The question addressed in this study was to investigate whether a mammalian pathogen taxonomically related to *Xenorhabdus* was able to substitute for or "hijack" the symbiotic relationship associating *Xenorhabdus* and *Steinernema* EPNs. To deal with this question, a laboratory experimental model was developed consisting in *Galleria mellonella* insect larvae, *Steinernema* EPNs with or without their natural *Xenorhabdus* symbiont and *Yersinia pseudotuberculosis*

brought artificially either in the gut of EPNs or in the haemocoel of the insect larva prior to infection.

The developed model demonstrated the capacity of EPNs to act as an efficient reservoir ensuring exponential multiplication, maintenance and dissemination of *Y. pseudotuberculosis*.

1. Introduction

Entomopathogenic nematodes (EPNs) are microscopic soil worms exclusively feeding on insect preys. They have the ability to cause death in a huge variety of insects, making them powerful candidate biopesticides in agriculture and horticulture (Brusselman et al., 2006; Wilson and Ivanova, 2004). EPNs owe their insecticidal properties to symbiotic bacteria belonging to two genera of *Enterobacteriaceae*, namely *Xenorhabdus* and *Photorhabdus*. These bacteria are hosted in the gastro-intestinal tract of the nematode – located in an intestinal receptacle in the case of *Xenorhabdus* (Poinar and Thomas, 1966) – at the infectious free-living stage, called infective juveniles (IJs). Upon invasion of an insect prey, the symbiotic bacteria are expelled from the IJ's digestive tract. These bacteria multiply in the insect haemocoel and release insecticidal toxins as well as degradative enzymes able to digest the insect macromolecules, thereby feeding their EPN partners which mature to the adult stage through 4 larval stages named J1 to J4 and undergo several reproduction cycles (Nick R Waterfield et al., 2009). Moreover, the symbiont prevents microbial competitors growth inside the insect's cadaver by releasing antibiotic and antifungal compounds (Li et al., 1997). After all insect macromolecules have been exhausted, a few symbiotic bacteria enter the intestinal tract of the mature IJs just before they emerge from the

dead larva and seek another prey (Emelianoff et al., 2008a; Goodrich-Blair and Clarke, 2007).

While a few EPNs are generally sufficient to kill an insect prey, up to half a million of IJs can emerge from a single infected host upon completion of their reproductive life cycle inside the insect cadaver (Forst et al., 1997). Each of these freshly emerged IJs is able to infect a new insect prey. IJs can survive in the soil for several months thanks to their protective cuticle and a huge lipid supply they can store (Hatab et al., 1998).

In 2008 Heermann and Fuchs have shown that *Photorhabdus luminescens*, the bacterial symbiont of *Heterorhabditis bacteriophora*, shares a number of unique genes with the taxonomically related, yet ecologically different, *Yersinia enterocolitica* (Heermann and Fuchs, 2008). The shared genes are for some of them clustered in so-called "High Pathogenicity Islands" described in *Enterobacteriaceae* including *Yersinia* (Schubert, 2004). Many of these genes are either involved in pathogenicity toward insects, like the insecticidal toxin complex (Tc) (Sheets et al., 2011), or in colonisation of eukaryotic cells, like the YplA phospholipase (Schmiel et al., 1998). The recently discovered type 6 secretion system (T6SS) involved in toxin secretion and in mutualism between bacteria (Jani and Cotter, 2010) is also conserved between *P. luminescens* and *Y. enterocolitica* (Heermann and Fuchs, 2008). Unlike *P. luminescens* which can cause casual infection in humans (Farmer et al., 1989a), *Y. enterocolitica* as well as *Y. pseudotuberculosis* are mammalian pathogens causing gastro-intestinal diseases in infected hosts. These two *Yersiniae* are regularly isolated from meat – especially pork meat – and root vegetables (Bari et al., 2011; Jalava et al., 2006). However, they have also been found in the gut lumen of adult flies and fly larvae, suggesting that they can use insects as passive vectors (Rahuma et al.,

2005; Zureck et al., 2000; Zurek et al., 2001). In addition, in vitro experiments have shown that both *Y. enterocolitica* and *Y. pseudotuberculosis* are able to colonize insect cells (Pinheiro and Ellar, 2007) and even to kill insect larvae like *Galleria mellonella* (Champion et al., 2009). It is well known that *Yersinia pestis*, the third mammalian pathogenic *Yersinia* and etiological agent of plague, is able to colonize insects since it uses fleas as vectors. Hinnebusch et al. demonstrated the essential implication of the *Yersinia* murine toxin (Ymt) in flea colonisation (Hinnebusch et al., 2002). Interestingly, it has been suggested that *Y. pestis* acquired *ymt* gene from *P. luminescens* or from a close relative (Duchaud et al., 2003). Moreover, *Photorhabdus asymbiotica* which can infect either insects or humans, possesses a plasmid related to pMT-1 found in *Y. pestis* (Wilkinson et al., 2009).

Besides their animal hosts, *Y. enterocolitica* as well as *Y. pseudotuberculosis* are commonly found in water, soil and vegetables (Bari et al., 2011; Buzoleva and Somov, 2003; Jalava et al., 2006). Several studies have shown that *Y. pestis* can also be found in soil (Ayyadurai et al., 2008; Eisen et al., 2008). Moreover, several experiments have highlighted the survival of *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* in free living soil amoeba (Lambrecht et al., 2013; Nikul'shin et al., 1992). Since pathogenic *Yersiniae* are able to persist in soil and are phylogenetically very close to the bacterial symbionts of EPNs, we wondered whether *Yersiniae* would be able to intrude the symbiotic relationship associating EPNs and their natural symbiont. In order to test this hypothesis, we used an experimental model consisting of insect larvae of the species *Galleria mellonella* used as prey for an African species of entomopathogenic *Steinernema* hosting its natural *Xenorhabdus* symbiont as well as a *Y. pseudotuberculosis* field isolate naturally resistant to the anti-microbial compounds produced by

Xenorhabdus. We show that *Y. pseudotuberculosis* can be successfully transmitted by the EPN carrier inside an insect larva in which it persists and multiplies. Moreover, EPNs emerging from the insect cadaver after 10 to 15 days where found to host large numbers of *Y. pseudotuberculosis* cells in their gastro-intestinal tract. These EPNs were in turn able to transmit *Y. pseudotuberculosis* to a new insect larva and so on for at least 7 successive infectious cycles (14 weeks). If they turn out to have an ecological significance, these findings may reveal an unexpected biotic reservoir for the long-term persistence and dissemination of pathogenic Yersiniae in the environment.

2. Material and Methods

2.1. Bacterial strains, plasmids and growth conditions

Enterobacteriaceae were grown in LB liquid broth with strong agitation (150rpm) or on LB agar or McConkey agar plates. The NBTA plates (nutrient agar supplemented with 25 mg l^{-1} bromothymol blue and 40 mg l^{-1} triphenyltetrazolium chloride) (Akhurst, 1980) were also used to check the form I of the *Xenorhabdus* species used in this study. The incubation temperature was 37°C except for *Yersiniae* and *Xenorhabdus* sp. which were grown at 28°C. Antibiotics were added at the following concentrations: Kanamycin (Km): 30µg ml^{-1}, Nalidixic acid (Nal): 25µg ml^{-1}, Streptomycin (Sm): 50µg ml^{-1}; Ampicillin (Ap): 100µg ml^{-1}. Nalidixic-acid resistant (Nal^R) bacteria were obtained in three consecutive steps by plating 10^7 to 10^9 CFU per agar plate supplemented with increasing concentrations of Nalidixic acid (5µg/ml; 20µg/ml; 50µg/ml). Bacterial strains used in this study are listed in Table 4.

Some *Enterobacteria*, listed in Table 4, were fluorescently labelled with GFP-mut2 (Cormack et al., 1996) using a mini-Tn*5* transposon (Reznikoff, 2008). Mini-transposon labelling was conducted by conjugating a nalidixic-acid resistant variant of the target bacterium with *E. coli* S17/1 λpir hosting a transposon delivery suicide vector (Miller and Mekalanos, 1988). Transconjugants were isolated on selective agar plates and tested for GFP fluorescence. Integration of *gfp-mut2* was further confirmed by PCR with primers mut2-GFP_F (GGG ATC TTT CGA AAG GGC AGA TTG TGT GG) and mut2-GFP_R (GGA GAG GGT GAA GGT GAT GCA ACA TAC GG). The size of the amplified fragment was 543 bp. For dual labelling experiments, the *gfp-mut2* gene of *Y. pseudotuberculosis* 4N1G was substituted for *mCherry*, encoding a red-fluorescent protein, by allelic exchange. The replacement cassette consisted in *mCherry* flanked by the beginning and the end of the *gfp-mut2* nucleotide sequence. The upstream and downstream flanking parts consisted of 244 and 223 base pairs of *gfp-mut2*, respectively, obtained by PCR amplification. A ribosome binding site was added upstream of the *mCherry* open reading frame to ensure optimal translation. The replacement cassette was cloned into the mobilizable suicide vector pKNG101, which confers resistance to streptomycin and carries the counter-selectable marker *sacBR* (Kaniga et al., 1991). The recombinant suicide plasmid termed pSGCG was then transferred to *Y. pseudotuberculosis* 4N1G from the conjugative strain *E. coli* SM10λpir. Allelic exchange was conducted in two steps. Initial integration of pSGCG was first selected on specific agar plates containing streptomycin (100μg ml^{-1}). After purification of the recombinant strain, allelic exchange was selected on agar plates containing sucrose (100μg ml^{-1}) and recombinant colonies

expressing mCherry but not GFP were controlled by both epifluorescence microscopy and PCR.

2.2. Mini-Tn5 transposon insertion mapping

The mini-Tn*5* transposon used here to tag the *Yersinia pseudotuberculosis* derivatives 4N1G and 4N1C was mapped by TAIL-PCR using the method of Liu and Wittier (Liu and Whittier, 1995) and by sequencing of the amplified fragment. The mini-Tn*5*-gfp was found inserted in the chromosome at codon 60 of the fimbrial A protein gene in the same transcriptional orientation (ORF YPK_0694 as described in the annotated genome of *Y. pseudotuberculosis* YPIII, Accession number NC_010465.1). The transposon-specific primers used for TAIL-PCR were the following: SP1: CGC GAA AGT AGT GAC AAG TGT TGG CCA TGG; SP2: GTA TAA CAT GTC TTA TAC GCC CGT GTC AAC C; SP3: AGA TCC CCG GGT ACC GAG CTC GAA TTC GCG. The arbitrary degenerated (AD) primers used here were the same described by Liu and Whittier (Liu and Whittier, 1995). Final confirmation of the insertion point of the mini-Tn*5* transposon was obtained by amplifying chromosomal fragments covering part of the transposon and part of the fimbrial A protein gene using the PCR primers SP1, SP2 or SP3 together with the fimbrial-specific primer CCG GTT CTA TCA TTG AAG CAC CTT GTT C.

2.3. Growth and maintenance of nematodes

Steinernema sp. MW8B isolated from Tanzanian soil (Mwaitulo et al., 2011) was used as model nematode allover the experiments. *Steinernema* sp. MW8B is symbiotically associated with *Xenorhabdus* sp. strain TZ01. Nucleotide sequence of the TZ01 16S ribosomal RNA gene (GenBank accession JQ687358.1) is equally similar, though not 100%

Table 4: List of bacterial strains used in this study.

Strains	Origin (Reference)	Description
Escherichia coli S17-1λPir	NCCB * (Simon, R. et al., Biotechnol. (1983) 1: 784–791, McFarlane, G.J.B. et al, J. Microbiol. Methods (1987) 6: 301–305)[51,52]	λ lysogenic S17-1 derivative expressing the π protein required for replication of plasmids carrying *oriR6K*; Sm^R
E. coli 17WP	This work (de Lorenzo, V et al., J. Bacteriol. (1990) 172(11):6568–72)[53]	*E. coli* S17-1 λPir carrying pUT-miniTn5-*gfpmut2*, a transposon delivery suicide vector. GFP mini-transposon delivery strain; Sm^R/Ap^R/Km^R
E. coli SM10 λPir	(Miller & Mekalanos, J. Bacteriol. (1988) 170 (6):2575–83)[34]	λ lysogenic *E. coli* derivative expressing the π protein required for replication of plasmids carrying *oriR6K*; Km^R
E. coli 10WP	This work	*E. coli* SM10 λPir carrying *mCherry* flanked by *gfp-mut2* moieties and cloned into pKNG101. Fluorescence cassette replacement vector termed pSGCG; Km^R, Sm^R, *sacBR*$^+$
E. coli strain EC26-KH-2010	VAR[§]	Vero-toxigenic *Escherichia coli* of the O157 serogroup isolated from a Belgian calf in 2010
E. coli strain VT02	This work	Nalidixic-acid resistant mutant of EC26-KH-2010
E. coli strain VT03	This work	VT02 carrying a randomly inserted Gfpmut2-transposon; Nal^R/Km^R
Xenorhabdus sp. strain TZ01	Anne Laudisoit, This work (Mwaitulo et al., Int. J. Trop. Insect Sci. (2011) 26(4):214–226)[37]	Symbiotic bacterium retrieved from *Steinernema tanzaniensis* nematodes isolated from Tanzanian soil
X. sp. strain TZ02	This work	Nalidixic-acid resistant mutant of TZ01
X. sp. strain TZ03	This work	TZ02 carrying a randomly inserted Gfpmut2-transposon; Nal^R/Km^R
Yersinia pseudotuberculosis strain IP2777	Institut Pasteur Lille (Derbise et al., J. Infect. Dis. (2013) 207(10):1535–43)[54]	Human clinical isolate, serotype O1

Y. pseudotuberculosis strain 2008/04429	VAR[§]	Isolated from a rabbit cadaver (Belgium)
Y. pseudotuberculosis strain 4N1	This work	Nalidixic acid-resistant mutant of 2008/04429
Y. pseudotuberculosis strain 4N1G	This work	2008/00429 4N1 carrying a Gfpmut2-transposon inserted in the fimbrial A protein A gene (see M&M); Nal^R/Km^R
Y. pseudotuberculosis strain 4N1C	This work	4N1G with *mcherry* replacing *gfpmut2* in the mini-transposon following allelic exchange using pSGCG.
Y. enterocolitica strain VAR08/02	VAR[§]	Pig Isolate belonging to serotype O3
Y. enterocolitica strain YE02	This work	Nalidixic-acid resistant mutant of VAR08/02
Y. enterocolitica strain YE03	This work	YE02 carrying a randomly inserted Gfpmut2-transposon; Nal^R/Km^R
Salmonella enterica subsp. *enterica* sv. Enteritidis strain 2011/03561	VAR[§]	Field isolate of poultry origin (Belgium)
*S.*Enteritidis strain SE02	This work	Nalidixic-acid resistant mutant of 2011/03561
*S.*Enteritidis strain SE03	This work	SE02 carrying a randomly inserted Gfpmut2-transposon; Nal^R/Km^R
Serratia marcescens strain EE016	This work	Isolated from a *Steinernema* sp. MW8B-infected *G. melonella* larva
Ochrobactrum tritici strain EE10.1	This work	Isolated from a *Steinernema* sp. MW8B-infected *G. melonella* larva

* NCCB, The Netherlands Culture Collection of Bacteria, Utrecht, The Netherlands.

§ VAR, Veterinary and Agrochemical Research Center, Brussels, BELGIUM.

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identical, to that of *X. ehlersii*, *X. budapestensis*, *X. griffiniae* and *X. kozodoii*. Nematode stocks (Infective juvenile stage) were maintained by successive passages through the last larval stage of the greater wax moth, *Galleria mellonella*. Infection of the larvae was conducted by incubating 500 to 1000 *Steinernema* sp. MW8B IJs suspended in 1ml physiological water (NaCl 9g L^{-1}) with four to six larvae confined in a closed Petri dish. Upon emergence from the dead larvae which occurred after 10 ± 2 days later, IJs were collected and stored at room temperature in physiological water.

2.4. Galleria mellonella in vitro infection model

For the first infection cycle, 6 *G. mellonella* larvae were injected with 10^6 CFU of the studied bacterium (*Yersinia* sp., GFP-labelled or not) using sterile 1-ml syringes bearing 0.3 X 13mm needles (Becton Dickinson). Injection was performed on the side of the larvae at the basis of the 8th segment. After incubation with ±750 *Steinernema* sp. MW8B IJs (125 IJs/larva) associated with their natural symbiont *Xenorhabdus* sp. TZ01, *G. mellonella* larvae died at day 1 or 2 post-infection and new IJs, named IJs_1, emerged at day 10 ± 2. IJs_1 were collected and washed thrice with physiological water prior to a new infection cycle started by transferring these IJs_1 to plates containing naive (*Yersinia*-free) *G. mellonella* larvae. Ten days later, a new generation of IJs, named IJs_2, emerged from the dead larva and so on for up to 7 consecutive infection cycles. The *Xenorhabdus* symbiont remained associated with *Steinernema* sp. MW8B throughout all infection cycles.

2.5. Gnotoxenic EPN engineering

Axenic EPNs were obtained by manually collecting eggs from gravid *Steinernema* sp. MW8B females recovered from infected *G. mellonella* larvae prior to the term of the infectious cycle. Such axenic eggs (min 3,000) were surface sterilized with a fresh sterilization solution obtained by diluting 1ml of a 15% NaClO solution and 1ml of a 4M NaOH solution in 10ml of distilled water. The sterile axenic eggs were then suspended in YS liquid medium at 25°C during 3 days and checked for J1 larval stage development. YS medium was prepared by dissolving the following components in 1L of distilled water: 5g yeast extract (Oxoid, Basingstoke, United Kingdom); 5g NaCl (Merck, Darmstadt, Germany); 0.5g $NH_4H_2PO_4$ (Merck); 0.5g K_2HPO_4 (Merck); and 0.2g $MgSO_4.7H_2O$ (Merck). If no contaminants were present, IJ_1 were deposited onto a Wouts agar plate (Wouts, 1981) that had been freshly inoculated with 10^8 CFU of the target bacterium in the absence of selective antibiotics. In the following days, EPNs matured to the adult stage and completed their reproductive cycle. After one week, monoxenic EPNs (IJ stage) were collected in physiological water and stored for later *Galleria* infection experiments. A similar procedure was followed to engineer polyxenic EPNs, *i.e.* EPNs harbouring two or more bacterial species.

2.6. Microscopic observations

A 10-µl suspension of infective juveniles (IJs) was observed on a microscope slide (covered with a 18x18mm coverslip) with an epifluorescence optical microscope (Olympus BX-40-FX) with objectives 10x/0.25 (for EPNs) and 40x/0.75 (for EPNs and bacteria). Samples were observed under visible and UV light (Hg) adjusted for optimal GFP or mCherry fluorescence.

2.7. Bacterial counts

To quantify the amount of bacteria contained in one IJs pool, and to avoid any contamination (either from the passage in *G. mellonella* or from the environment), IJs were surface sterilized following a standardized procedure. In brief, IJs were immersed in a 1.5ml eppendorf for 3 minutes with 1ml of a sterilization solution (as described previously in the M&M) with gentle agitation. After 1min centrifugation at 4000rpm in a minicentrifuge, supernatant was discarded and IJs were rinsed thrice with physiological water (0.9% NaCl). Finally, surface sterilised IJs were crushed and plated on selective agar. The number of IJs present in the pool was estimated by microscopic counting of a representative sample (50µl). Alternatively, a non-sterile method was used to assess the number of targeted bacteria associated with the IJs: *G. mellonella* cadavers were rinsed with physiological water to collect the freshly emerged IJs in suspension. The number of IJs per larva was estimated by microscopical count on 50-µl drops from this suspension. Serial dilutions of the supernatant were then plated on selective agar medium and bacteria were enumerated.

2.8. Theoretical count of Y. pseudotuberculosis

In order to stress out the *Y. pseudotuberculosis* multiplication during the EPNs infection cycle, the theoretical counts (T_Y) that would be observed starting from the same inoculum if no bacterial division would occur have been calculated. For this calculation, theoretical volumes of 0.5ml (V_{Gm}) and 0.8nl (V_{IJ}) have been assigned per *G. mellonella* larva and *Steinernema* sp MW8B IJ, respectively, and a mean EPN emergence yield of 50,000 EPNs (N_{IJ}) per larva has been considered. It has been also assumed that 125 IJs infect a single *G.*

mellonella larva. The dilution factor (DF) is the ratio $V_{Gm}/(V_{IJ} \times N_{IJ})$. T_Y is calculated by dividing the number of *Y. pseudotuberculosis* CFUs infecting a larva by the DF.

2.9. Susceptibility testing towards Xenorhabdus sp. antimicrobial compounds

To estimate the resistance of enterobacteria towards *Xenorhabdus* sp. TZ01 antibiotic compound production, growth of the tested bacteria was monitored every 30 minutes during 6 hours by optical density (OD) measurement in the presence of *Xenorhabdus* culture extracts. For this purpose, LB liquid medium was inoculated with an inoculum of the target bacterium derived from a fresh culture to reach an initial OD_{600} of 0.05-0.1. Prior to inoculation, LB was supplemented with 4% or 8% (v/v) of 0.2-µm filtered supernatant of a 48h liquid culture of *X.* sp. TZ01 grown at 28°C with shaking at 150rpm (Cell Free Supernatant). *X.* sp. TZ01 liquid cultures used for supernatant preparation were stopped when OD_{600} reached 11-13. For growth curve analysis, OD_{600} was plotted into a log2 scale in order to obtain a linear graph for the exponential phase of the curve. The slope of this line (calculated with GraphPad Prism® 6) defines the growth rate of a given bacterium in the defined culture conditions.

3. Results

3.1. Yersinia pseudotuberculosis resists to antimicrobial compounds produced by Xenorhabdus sp.

Susceptibility of several strains of *Y. pseudotuberculosis*, pathogenic *E. coli*, *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*S.* Enteritidis) and *Serratia marcescens* towards antimicrobial

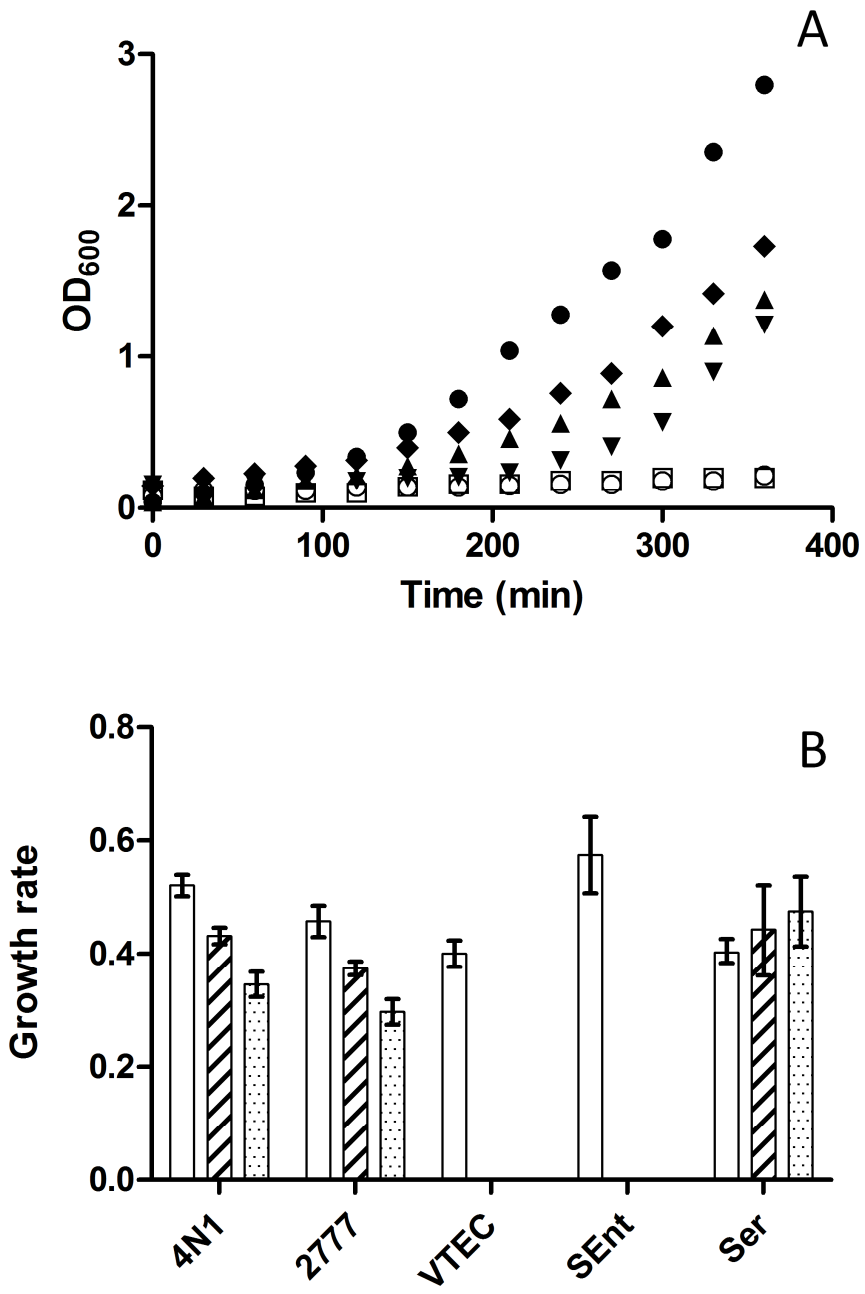

Figure 11: Susceptibility of various enterobacteria to antimicrobial substances produced by *X.* sp. TZ01.

A: Growth curves in liquid broth of *Y. pseudotuberculosis* 4N1 (closed circles), *Y. pseudotuberculosis* 4N1 supplemented with 8% of X. sp. TZ01 culture supernatant (closed triangles), *Y. pseudotuberculosis* IP2777 supplemented with 8% of *X.* sp. TZ01

culture supernatant (closed diamonds), *Serratia marcescens* EE016 supplemented with 8% of X. sp. TZ01 culture supernatant (closed upside down triangles), E. coli VT01 supplemented with 8% X. sp. TZ01 culture supernatant (open circles) and *S.* Enteritidis SE01 supplemented with 8% of X. sp. TZ01 culture supernatant (opened squares). OD_{600} values were plotted every 30 minutes during 6 hours.
B: Growth rates of *Y. pseudotuberculosis* 4N1 (4N1), *Y. pseudotuberculosis* IP2777 (2777), Vero-toxigenic *E. coli* VT01 (VTEC), *Salmonella* Enteritidis SE01 (SEnt) and *Serratia marcescens* EE016 (Ser) in liquid broth supplemented with either 0% (white bars), 4% (hatched bars) or 8% (dotted bars) of X. sp. TZ01 culture supernatant. Growth rates were calculated by plotting experimental OD_{600} values in log2 scale and taking the slope of the adjusted linear regression curve. Few or no growth was observed for Vero-toxigenic *E. coli* VT01 and *Salmonella* Enteritidis SE01 grown with either 4% or 8% of X. sp. TZ01 culture supernatant. CFUs per IJ decreased by 41%. However, quantitative results obtained for IJs_4 at week 3 PE and IJs_1 at day 0 PE are comparable.

compounds produced by *X.* sp. TZ01 was tested by growth curve analysis. Growth of *Y. pseudotuberculosis* (strains 4N1 and IP2777) was slightly delayed with 4% *X.* sp. TZ01 supernatant compared to a control growth without *X.* sp. TZ01 supernatant, but subsequent growth was merely unaffected when up to 8% *X.* sp. TZ01 supernatant was added. Likewise, *S. marcescens* EE016 was characterized by a delayed growth while its capacity to grow with up to 8% *X.* sp. TZ01 supernatant was unaffected. To the contrary, both *S.* Enteritidis SE01 and Vero-toxigenic *E. coli* VT01 strains were drastically inhibited with 4% *X.* sp. TZ01 supernatant (data not showed) and totally unable to grow with 8% added supernatant (Figure 11A). Growth rates of *Y. pseudotuberculosis* 4N1 and IP2777 were both slightly affected when *X.* sp. TZ01 supernatant was added. At 4% supernatant, slopes slightly decreased by about 1/5 for *Y. pseudotuberculosis* 4N1 and IP2777. The effect on the slopes doubled when 8% of *X.* sp. TZ01 supernatant was added. *S. marcescens* EE016 growth was unaffected with either 4% or 8% added *X.* sp. TZ01 supernatant (Figure 11B).

Aiming to assess the susceptibility of *Y. pestis*, five field isolated *Y. pestis* strains (Table 5) were tested towards antimicrobials secreted by *Xenorhabdus* sp. TZ01 following an adapted procedure described in section 2.9. These experiments were conducted in the biosafety level 3 laboratory of VAR. Liquid cultures were maintained at room temperature (between 24.6°C and 25.2°C) with strong agitation (150 rpm). Cultures were supplemented with 8% (v/v) of *Xenorhabdus* sp. TZ01 supernatant prior to inoculation. To observe any effect of this supernatant on *Y. pestis* growth, OD_{600} were monitored thrice at the inoculation point, at 6 hours post inoculation and at 24h post inoculation. With only 3 experimental measurements per assay, neither growth curves nor growth rates could be calculated. The results presented here are just an indication of the ability of *Y. pestis* to survive to antimicrobial compounds released by *Xenorhabdus* sp. TZ01. A susceptible *E. coli* strain was used as positive control.

Table 5: List of *Y. pestis* strains used for susceptibility tests

Strain name	**Origin**
Y. pestis NCTC 10030	Nairobi (Kenya)
Y. pestis NCTC 5924	Java (Indonesia)
Y. pestis NCTC 570	Bombay (India)
Y. pestis RDC 01	Katanga (DRC[1])
Y. pestis RDC 09	Ahulogo (DRC[1])

[1] Democratic Republic of Congo

Two profiles can be distinguished among the five tested strains. A first profile includes *Y. pestis* NCTC 5924 and *Y. pestis* NCTC 570, termed the Asian group and a second profile including *Y. pestis* NCTC 10030, *Y. pestis* RDC 01 and *Y. pestis* RDC 09, termed the African group.

On one hand, the Asian group growth is totally inhibited with 8% of *X*. sp TZ01 supernatant even at 24h post inoculation while the *E. coli* TOP10 control strain finally multiplied to reach the exponential phase 24h post inoculation. On the other hand, the growth of the African group is still inhibited at 6h post inoculation but bacteria reached the exponential phase 24h post inoculation (Figure 12).

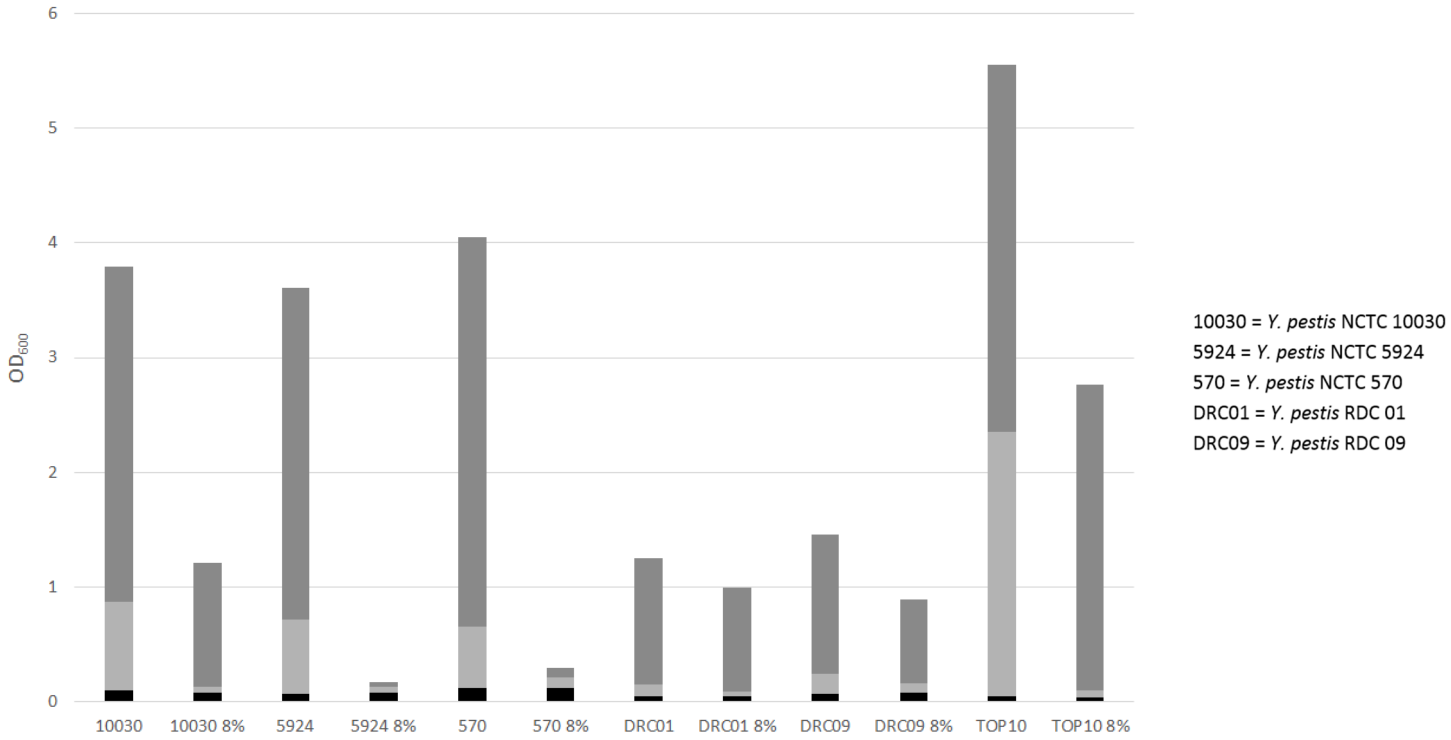

Figure 12: Susceptibility of five *Y. pestis* strains towards *X.* sp TZ01 antimicrobials

OD_{600} are measured at inoculation time (black), at 6h post inoculation (light grey) and at 24h post inoculation (dark grey) either with (8%) or without addition of *X.* sp. TZ01 supernatant.

3.2. *Yersinia pseudotuberculosis colonizes the gastro-intestinal tract of EPNs and survives long-term EPN storage.*

To assess the ability of *Y. pseudotuberculosis* to colonize *Steinernema* sp. MW8B, 7 independent *Galleria mellonella* infection experiments were conducted with a *Y. pseudotuberculosis* GFPmut2-labelled strain (4N1G strain) (Table 6).

In all experiments, *Steinernema* sp. MW8B IJs_1 exhibited GFPmut2 fluorescence along the entire length of their gut (Figure 13A.1). However, in 2 out of 7 experiments (29%), IJs_1 emerged from *Y. pseudotuberculosis* 4N1G-infected *G. mellonella* larvae failed to invade and kill new naive *G. mellonella* larvae in spite of the fact that they were massively colonized by *Y. pseudotuberculosis* 4N1G as attested by the bright GFP fluorescence they displayed. In 2 out of 7 experiments (29%), IJs that were both fluorescent and infective emerged from dead *G. mellonella* cadavers after 4 consecutive infection cycles (Figure 13A.2). One experiment (14%) led to the emergence of fluorescent / infective IJs after the fifth infection cycle and were so even after 7 consecutive infection cycles which lasted 14 weeks (Figure 13A.3).

Directly after the first emergence, freshly emerged IJs_1 were stored at 4°C, 16°C and 28°C in physiological water. IJs_1 were observed in epifluorescence microscopy to monitor the presence of *Y. pseudotuberculosis* 4N1G. These observations were made every day during the first week post emergence (PE) then once a week during 13 weeks. At 4°C, stored IJs_1 did not survive a week and were all dead by day 8 PE (Figure 13B.1 and B.2).

Table 6: Summary of *G. mellonella* infection experiments

Infection Cycle	IJ emergence	IJ fluorescence	IJ infectivity	IJs/larva	*Yp* CFU/IJ	Total *Yp* count
1	7/7	7/7	5/7	50000 +/– 7500	$5{,}0 \times 10^3$ +/– $0{,}8 \times 10^3$	$2{,}5 \times 10^8$ +/– $0{,}3 \times 10^8$
2	5/7	3/7	4/7	ND	ND	ND
3	4/7	2/7	3/7	ND	ND	ND
4	3/7	2/7	3/7	40800 +/– 6366	$8{,}6 \times 10^3$ +/– $1{,}6 \times 10^3$	$3{,}5 \times 10^8$ +/– $0{,}5 \times 10^8$
5	3/7	1/7	2/7	ND	ND	ND
6	2/7	1/7	1/7	ND	ND	ND
7	1/7	1/7	NA	1022 +/– 247	$5{,}6 \times 10^3$ +/– $1{,}8 \times 10^3$	$5{,}7 \times 10^6$ +/– $1{,}4 \times 10^6$

Seven *G. mellonella* larvae were injected with 10^6 CFU of the GFP-labelled *Y. pseudotuberculosis* strain 4N1G (*Yp*) and incubated with ca. 125 *Steinernema* sp. MW8B nematodes (Infective Juvenile stage, IJ) associated with their natural *Xenorhabdus* sp. TZ01 symbiont as described in M&M. After completion of the first infectious cycle, *Steinernema* sp. MW8B progeny emerging from one death larva was collected, characterized according to several parameters and used to infect naïve (*Yp*-free) *G. mellonella* larvae thereby initiating a new infection cycle, and so on for 7 successive cycles. Column 2–4, number of experiments in which emerged IJs fitted the property featured on the top row; column 5, mean number of IJs emerged from one dead larva; column 6, mean count of *Yp* CFU retrieved from one crushed IJ; column 7, total count of *Steinernema* sp. MW8B-associated *Yp* CFU generated from one *G. mellonella* larva after successful cycle completion. ND, not determined; NA, not available. The *Xenorhabdus* sp. TZ01 symbiont was still present after each successful cycle completion.

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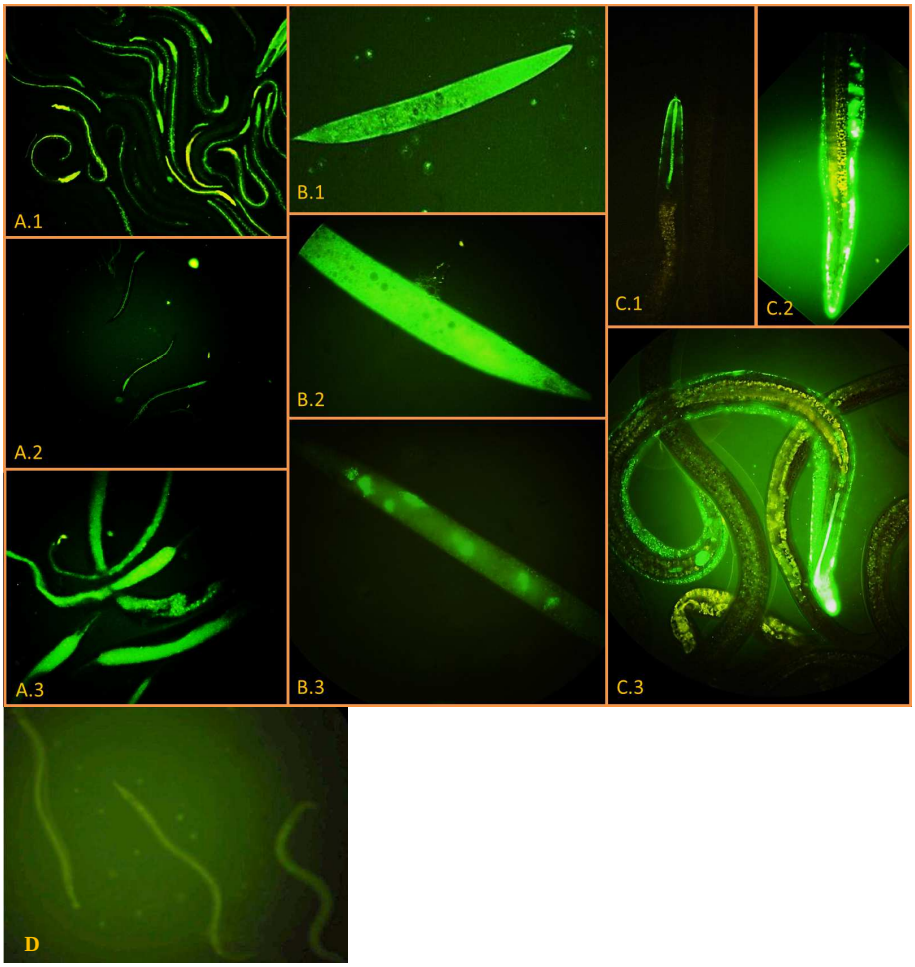

Figure 13: Epifluorescence microscope pictures of GFP-labelled *Y. pseudotuberculosis* 4N1G in *Steinernema* sp. MW8B EPNs.

A. EPNs emerging from dead moth larvae after 1 (A.1), 4 (A.2) and 7 (A.3) consecutive infection cycles (100× magnification).
B. IJs collected after the first infection cycle and stored at 4°C in physiological water for either 8 (B.1, B.2) or 42 days (B.3) (400× magnification).
C. IJs collected after the first infection cycle and stored at 28°C in physiological water for 98 days. C.1, enlarged view of the mouth; C.2, enlarged view of the anus; C.3, whole IJ body (400× magnification, 800× magnification for enlarged view)
D. Uninfected IJs

Nevertheless, *Y. pseudotuberculosis* 4N1G was still alive – and did even multiply slowly inside the IJs cadavers – since GFP fluorescence was still observed 6 weeks PE (Figure 13B.3). No differences were observed between IJs_1 stored either at 16°C or at 28°C. In these samples, microscopic observations showed that *Y. pseudotuberculosis* 4N1G was still present inside IJs_1 of *Steinernema* sp. MW8B, either in the gut or in the inter-cuticular space, after 14 weeks of storage at either 16°C or 28°C (Figure 13C).

To check the ability of *Y. pseudotuberculosis* 4N1G to remain associated with *Steinernema* sp. MW8B after several infection cycles, IJs_4 were also kept at 28°C. At 3 weeks PE, 23 +/- 3 IJs_4 were crushed and counted on selective agar plates. An average of 5.0 x 10^3 CFUs of *Y. pseudotuberculosis* 4N1G per IJs_4 was measured. Compared to IJs_4 at 0 day PE (8.6 x 10^3 CFU/IJs_4), the number of *Y. pseudotuberculosis* 4N1G per IJs_4 was measured. Compared to IJs_4 at 0 day PE (8.6 × 103 CFU/IJs_4), the number of *Y. pseudotuberculosis* 4N1G CFUs per IJ decreased by 41%. However, quantitative results obtained for IJs_4 at week 3 PE and IJs_1 at day 0 PE are comparable.

Similar *G. mellonella* infection experiments were conducted three times with a GFPmut2-labelled *Y. enterocolitica* O:3 strain (YE03). This strain is more sensitive towards antimicrobials produced by *X.* sp. since *Y. enterocolitica* O:3's growth is totally inhibited with the presence of 8% of *X.* sp. supernatant (data not shown). Microscopic observations showed EPN colonization by *Y. enterocolitica* YE03 during at least 2 consecutive infection cycles for one out of the three experiments conducted. Confocal microscopic observations localized *Y. enterocolitica* YE03 in the gut lumen after 2 infection cycles (Figure 14). However, no GFPmut2 fluorescence was observed after 3 consecutive infection cycles with *Y. enterocolitica* YE03.

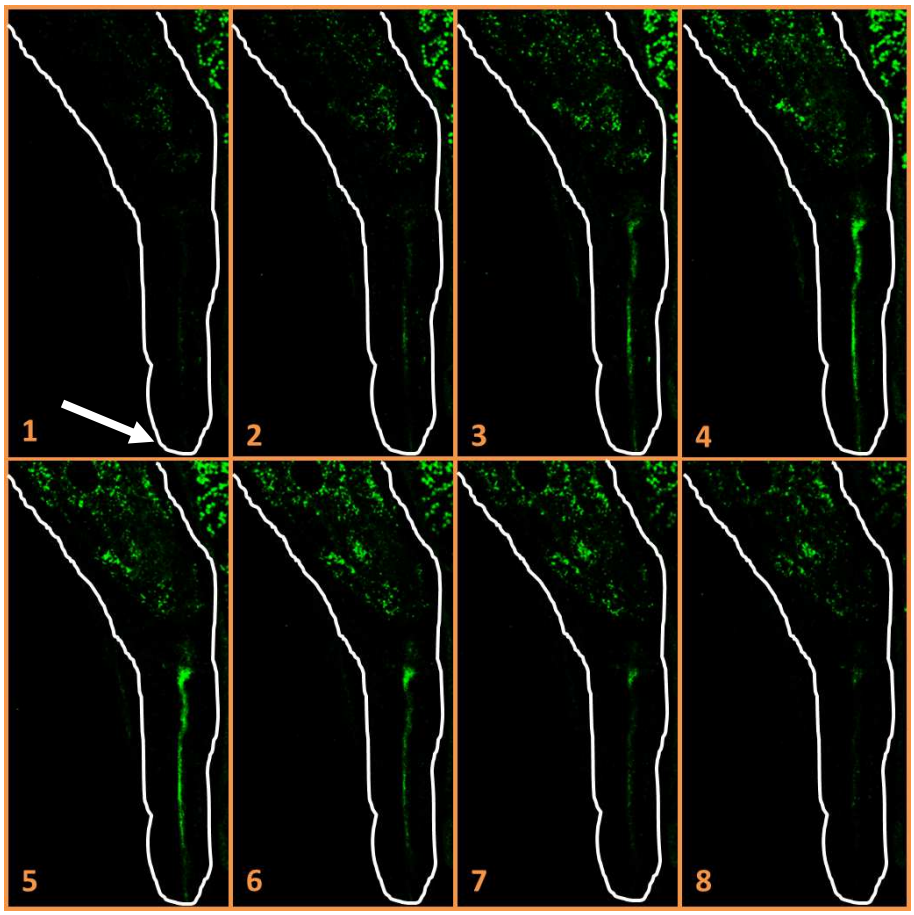

Figure 14: Localization of *Y. enterocolitica* YE03 in *Steinernema* sp. MW8B EPNs emerged from an infected larva.

Confocal microscope slides in Z-axis (numbered from 1 to 8) of a *Steinernema* sp. MW8B EPN colonized by *Y. enterocolitica* YE03 emerged from the second infection cycle. GFP-labeled bacteria localize in the mouth (showed by the white arrow) and in the gut lumen. EPN borders are drawn in white (800× magnification)

GFPmut2-labelled *Escherichia coli* VT03 (vero-toxigenic O157 strains), GFPmut2-labelled *Salmonella* Enteritidis SE03 and an unlabelled tetracycline resistant *S. marcescens* EE016 strain were subjected to similar *G. mellonella* infection cycle experiments. None of these 4 *Enterobacteriaceae* demonstrated *Steinernema* sp. MW8B colonisation

capacity. This was evidenced by the lack of GFPmut2 fluorescence in IJs_1 in the *E. coli* VT03 and *S.* Enteritidis SE03 experiments. No single IJ_1 emerged from *G. mellonella* larvae injected with *S. marcescens* EE016 while emergence was observed when the insects were not injected.

3.3. EPNs support dramatic multiplication and dissemination of Yersinia pseudotuberculosis.

To confirm quantitatively the maintenance of *Y. pseudotuberculosis* 4N1G in the experimental model, CFU counts were determined at different time points (Table 6). After the first infection cycle, an average of 5.0 x 10^3 *Y. pseudotuberculosis* 4N1G CFUs per *Steinernema* sp. MW8B IJ were found. Similar counts were determined during 7 consecutive infection cycles, with an average of 8.6 x 10^3 CFUs of *Y. pseudotuberculosis* 4N1G per IJ still found after the 4^{th} infection cycle and 5.6 x 10^3 CFUs after the 7^{th} infection cycle (Table 6). Knowing the number of CFU per IJ and the number of IJs emerged from dead larvae, we calculated the total increase in *Y. pseudotuberculosis* 4N1G counts after the various infection cycles. Starting with 1.9 x 10^6 CFUs of *Y. pseudotuberculosis* 4N1G directly injected in the larva, *Y. pseudotuberculosis* 4N1G counts after one cycle increased by two orders of magnitude and reached 2.5 x 10^8 CFUs. These counts were similar after the fourth infection cycle (3.5 x 10^8 CFU) and started to decrease from the 7^{th} infection cycle (5.67 x 10^6 CFU). This confirms our model predictions which suggest that in the absence of active multiplication, *Y. pseudotuberculosis* 4N1G counts would drastically decrease and would become undetectable after two infection cycles (Figure 15). The experimental counts hence reflect an active multiplication of *Y. pseudotuberculosis* 4N1G in the studied laboratory model.

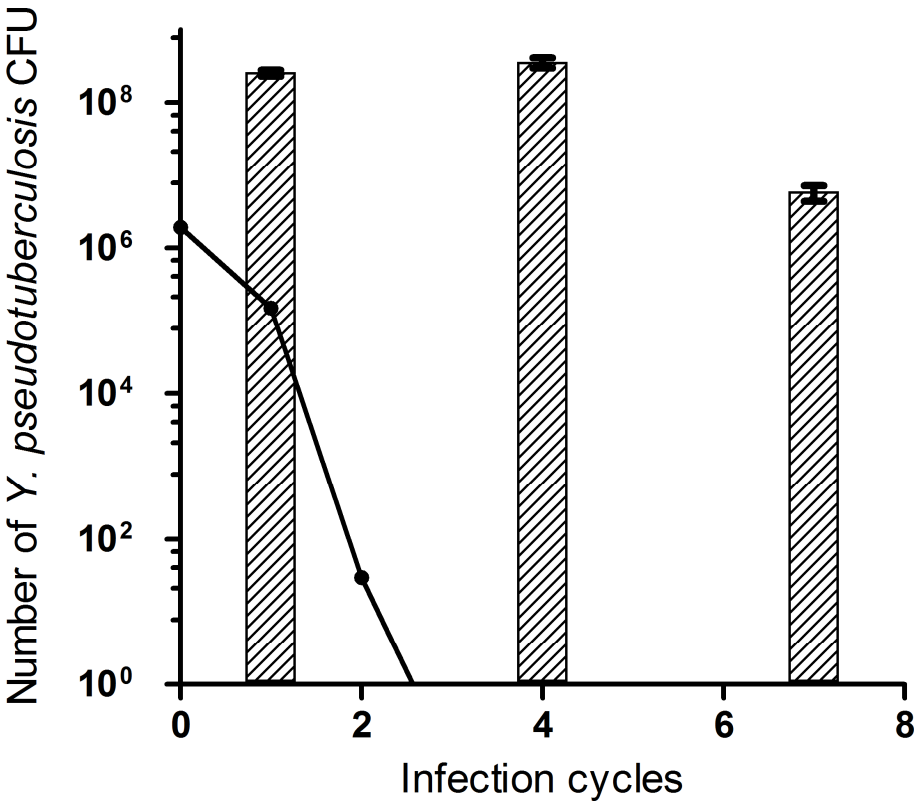

Figure 15: Growth of *Y. pseudotuberculosis* 4N1G during EPN's infection cycles. The hatched bars show the total counts of *Y. pseudotuberculosis* 4N1G CFUs retrieved from IJs emerged from a dead moth larva after 1, 4 and 7 consecutive infection cycles (data from table 2). The straight line shows the theoretical counts that would be observed starting from the same inoculum if no bacterial division would occur. For this calculation, theoretical volumes of 0.5ml and 0.8nl have been assigned per G. mellonella larva and *Steinernema* sp. MW8B IJ, respectively, and a mean EPN emergence yield of 50,000 EPNs per larva has been considered (see M&M)

3.4. *Yersinia pseudotuberculosis* cannot replace *Xenorhabdus* sp. TZ01 as EPN symbiont.

After having demonstrated the colonisation and multiplication capacity of *Y. pseudotuberculosis* 4N1G in the gut of *Steinernema* sp. MW8B IJs, we wondered whether *Y. pseudotuberculosis* 4N1G could substitute for *X.* sp. TZ01 as a bacterial symbiont in this EPN species. To address this question, we obtained axenic *Steinernema* sp. MW8B EPNs by collecting surface sterilised eggs from gravid females. Prior to *G. mellonella* infection, axenic EPNs were incubated with the mCherry-labelled *Y. pseudotuberculosis* 4N1C strain on Wouts Agar plates in order to obtain IJs exclusively colonised by *Y. pseudotuberculosis* 4N1C. A pool of such "monoxenic" IJs displaying red fluorescence (Figure 16A) was divided into 4 equal groups. Two groups were incubated separately with 6 *G. mellonella* larvae in empty containers. One group was deposited onto a sterile Wouts Agar plate with 4 *G. mellonella* larvae and the last group was deposited onto a Wouts Agar plate without any larva. In a empty container, normal uninfected IJs were incubated with 6 *G. mellonella* larvae as a positive control.

At day 3 post infection (PI), *G. mellonella* larvae grown on Wouts Agar and the ones of the positive control were found dead. At day 8 PI, all *G. mellonella* were dead. Three *G. mellonella* larvae recovered from Wouts agar plates showed emergence of EPNs. The emerged EPNs displayed no mCherry fluorescence when observed microscopically. When crushed and plated onto selective agar plates, no *Y. pseudotuberculosis* 4N1C could be retrieved from these EPNs neither. At day 10 PI, emergence of EPNs could be observed in 2 larvae incubated in empty containers, but again none of the emerged EPNs

exhibited red fluorescence and no *Y. pseudotuberculosis* 4N1C could be retrieved after EPN crushing and plating on selective agar. In contrast, EPNs grown freely on Wouts agar still exhibited red mCherry fluorescence 10 days after plating. The same experiment was conducted with the GFPmut2-labelled *X.* sp. TZ03. Microscopic observations showed not only that *X.* sp. TZ03 was able to colonise the symbiotic vesicle of *Steinernema* sp. MW8B axenic EPNs (Figure 16B), but also that *X.* sp. TZ03 maintained in its host after 2 consecutive cycles (Figure 16C) and probably much more (not tested).

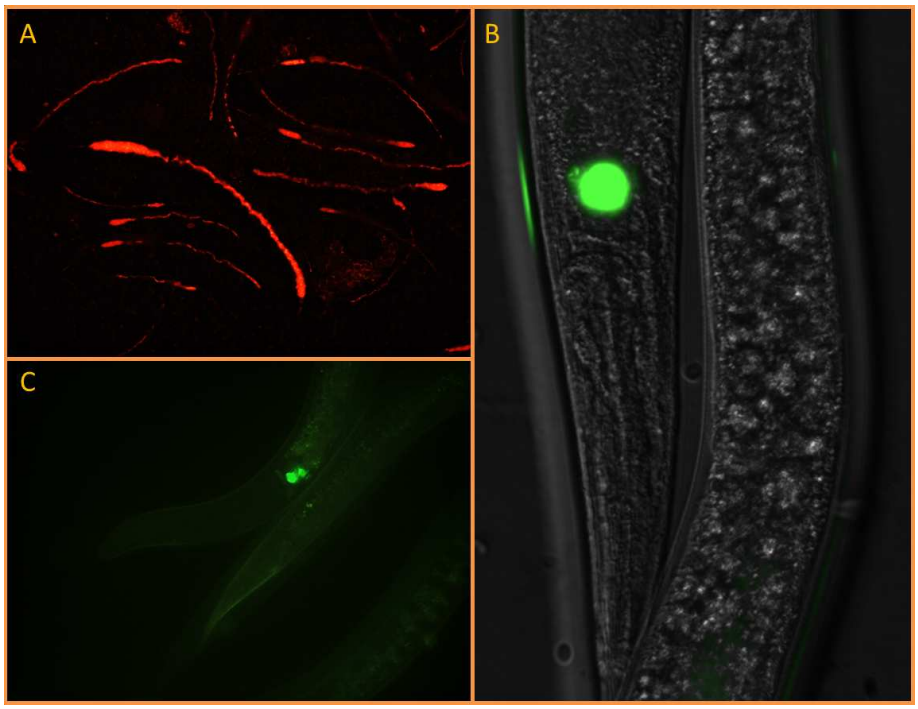

Figure 16: Differential localization of *Y. pseudotuberculosis* 4N1C and *X.* sp. TZ03 in *Steinernema* sp. MW8B nematodes.

Epifluorescence microscope pictures showing axenic EPNs artificially fed on (A) plate-grown red fluorescent *Y. pseudotuberculosis* 4N1C localizing in the gut (100× magnification) or (B) plate-grown green fluorescent *X.* sp. TZ03 localizing in a symbiotic vesicle (400× magnification). The latter was still localized in the symbiotic vesicle after 2 consecutive infection cycles on *G. mellonella* larvae (C) (800× magnification)

4. Discussion, Conclusion, and Perspectives

Compared to other enterobacteriaceae tested so far, the capacity of *Y. pseudotuberculosis* 4N1 to colonize *Steinernema* sp. MW8B is remarkably efficient and suggests that a number of biological functions required for its successful dissemination through this host during and between infection cycles are present and functional in this bacterium. We showed that enterobacteria sensitive to the antibiotics secreted by *Xenorhabdus.* sp. TZ01 have no ability to colonize the EPN gut. Two strains of *Y. pseudotuberculosis* (4N1 and IP2777) as well as one *S. marcescens* strain (EE016) isolated from a *Steinernema* sp. MW8B-infected *G. mellonella* larva were found naturally resistant to *X.* sp. TZ01 secreted antibiotics and were tested for their ability to colonize *Steinernema* sp. MW8B EPNs with the model developed herewith. The growth rate of both *Y. pseudotuberculosis* strains was slightly affected by the presence of *X.* sp. TZ01 supernatant, while the *S. marcescens* EE016 was not. Likewise, *Ochrobactrum tritici* strain EE10.1 isolated from a *Steinernema* sp. MW8B-infected *G. mellonella* larva in our laboratory displayed a similar capacity to resist to *X.* sp. TZ01 antibiotics (data not shown). Despite this capacity, *S. marcescens* EE016 was unable to sustain EPNs life cycle completion since no IJ emergence occurred from *S. marcescens*-injected *G. mellonella* larvae. This suggests that *Serratia* and *Ochrobactrum* may accidentally reach the gut of *Steinernema* sp. MW8B but are unlikely able to colonize and multiply within the EPN gut as *Yersinia pseudotuberculosis* does. It has been shown that *Serratia marcescens* uses the type VI secretion system to neutralize bacterial competitors (Murdoch et al., 2011). This competition, at the 10^6 CFUs injection level, may impair *Xenorhabdus* growth in *G. mellonella* larvae injected with *S. marcescens*. This could explain why *Steinernema* sp. MW8B cannot complete its reproductive

cycle within *S. marcescens*-infected *G. mellonella*. *S. marcescens* has been shown to be pathogenic towards the free-living nematode *Caenorhabditis elegans* but beneficial to the entomopathogenic nematode *C. briggsae* (Lancaster et al., 2012; Mallo et al., 2002). Zhang et al. reported the isolation of a new *Serratia* species (*S. nematodiphila*) from the EPN species *Heterorhabditidoides chongmingensis* and proposed that *S. nematodiphila* may have evolved to a symbiotic species, possibly after horizontal gene transfer (Zhang et al., 2009, 2008). Dixenic associations have been described, such as *P. luminescens* and *Ochrobactrum spp*. found together in tropical species of *Heterorhabditis* (Babic et al., 2000). Genomic comparison between *S. nematodiphila* and other (non-symbiotic) *Serratia spp*. could provide interesting insights in the discovery of genes involved in the symbiotic association with EPNs.

Concerning the suscpetibility of Y. pestis towards X. sp. Antibiotics, the observations suggest that *X*. sp. TZ01 supernatant has a bactericidal effect on the Asian strains while it has a bacteriostatic effect both on the African group and on the *E. coli* TOP10, only causing a delay in their growth. Continuous OD_{600} monitoring is required to evaluate more accurately the impact of *X*. sp TZ01 supernatant on *Y. pestis* growth and would allow comparison with *Y. pseudotuberculosis* resistance profile analyzed previously. Nevertheless, these preliminary results suggest that none of the *Y. pestis* strains tested here seem to be a good candidate to grow simultaneously with *Xenorhabdus* sp. within an insect cadaver.

In this study we showed that *Y. pseudotuberculosis* 4N1G is able to colonize and maintain for several generations inside a *Steinernema* species for long-term periods (14 weeks). Quantitative data showed that EPNs support efficient multiplication of *Y. pseudotuberculosis*

4N1G during this period. Indeed, counts of *Y. pseudotuberculosis* CFUs carried away by EPNs emerged from dead larvae are roughly multiplied by a factor 10^3 at the term of each infection cycle, a number which is probably underestimated as it does not take into account *Y. pseudotuberculosis* bacteria left over in the dead cadaver. *Y. pseudotuberculosis* 4N1G colonizes mainly the gut of *Steinernema* sp. MW8B but can be found in the inter-cuticular space as well after 3-month storage in physiological water. The localisation of *Y. pseudotuberculosis* 4N1G in *Steinernema* sp. MW8B IJs' gut is quite different from the normal localisation of the symbiotic *Xenorhabdus* sp. TZ03. Indeed, the natural niche of *Xenorhabdus* inside its *Steinernema* host - before infecting an insect prey - is a so-called symbiotic vesicle located along and separated from the EPN gut (Poinar and Thomas, 1966). Our observations on EPN colonization are consistent with axenic EPNs experiments, which demonstrated that *Y. pseudotuberculosis* 4N1G does not replace the *X.* sp. TZ01 symbiont during EPN infection cycle but more likely hijack the symbiotic relationship between *Xenorhabdus* and EPNs. Indeed, when the natural symbiont of *Steinernema* sp. MW8B is absent, EPNs colonized by *Y. pseudotuberculosis* 4N1C alone are unable to develop properly in a *G. mellonella* larva. In control experiments where axenic EPNs are supplemented with GFP-labelled *X.* sp. TZ03, EPNs recover their ability to indefinitely multiply and feed on *G. mellonella* larvae. Moreover, published results showed that IJs colonized by both *X.* sp. and *Y. pseudotuberculosis* strains labelled with two different fluorescent protein markers do contain both bacteria (Gengler et al., 2015a). The impact of *Y. pseudotuberculosis* on the EPN fitness was not determined. However, EPNs were grazed and complete their cycle on *Y. pseudotuberculosis* lawn. Although the mutiplication on *Y.*

pseudotuberculosis lawn qualitatively seemed to be less efficient than on *Xenorhabdus* lawn, the fact that EPNs are able to complete their cycle indicates that Y. pseudotuberculosis does not kill EPNs.

The mini-Tn*5* transposon used to tag *Y. pseudotuberculosis* 4N1G and 4N1C in our experiments was mapped in the fimbrial A protein gene. This gene is found in two intact copies in the *Y. pseudotuberculosis* genome meaning that the protein is probably still expressed – though not in the same level – in the GFP-tagged strains. Fimbrial proteins are known to act as colonization factors for *Y. pseudotuberculosis* and are involved in the attachment to epithelial cells (Collyn et al., 2002). The capacity of the 4N1G and 4N1C tagged strains to colonise the EPN's gut, as demonstrated throughout our study, argue in favour of a non-detrimental effect of the transposon insertion compared to the wild-type strain.

Interestingly, it has been shown that *Y. pseudotuberculosis* and *Y. pestis* – the etiological agent of plague – can infect or form a biofilm mainly around the head of *Caenorhabditis elegans*, a well-studied nematode laboratory model (Darby et al., 2002; Joshua et al., 2003; Tan and Darby, 2004). Given the fact that *Y. pestis* evolved quite recently from *Y. pseudotuberculosis* (Achtman et al., 1999), it would be interesting to know whether *Y. pestis* can also resist to antimicrobial substances produced by *Xenorhabdus* / *Photorhabdus* spp. and colonize EPNs. If these findings turn to have an environmental significance, it would provide new insights in the understanding of long-term persistence of *Y. pestis* in plague endemic areas worldwide (Bertherat et al., 2007; Eisen et al., 2008).

Future work should focus on the identification of *Yersinia* genetic determinants required to colonize and maintain inside EPNs.

Several genes shared by *Yersinia* and the EPN's natural symbionts are good candidates to play this role such as the phospholipase A encoded by *yplA*, structural genes of the type 6 secretion system (T6SS) and possibly others (Heermann and Fuchs, 2008). Likewise, genome comparisons between *Serratia*, *Ochrobactrum*, *Yersinia* and *Xenorhabdus* should help deciphering the critical genetic determinants required for EPN colonization.

5. Acknowledgment

We thank Dr. Ralf-Udo Ehlers (Kiel University, Germany) for teaching the methodology and tips for proper axenic EPNs preparation, Dr. Guy R. Cornelis (FUNDP, Namur, Belgium) for his gift of *E. coli* SM10 ®Pir and pKNG101, Dr. Florent Sebbane (Institut Pasteur de Lille, France) for his gift of *Y. pseudotuberculosis* IP2777, Michaël Abraham (VAR) for his help in the GFP-labeling of enterobacteria used in this study and Mieke Van Hessche and Jordane Lebrun (VAR) for susceptibility testing experiments.

Chapter 3 – Study of *Yersinia pseudotuberculosis* genetic determinants involved in *Steinernema* colonization

In the previous chapters, it has been shown that *Y. pseudotuberculosis* was able to colonize *Steinernema* nematodes. This chapter focuses on three *Y. pseudotuberculosis* genes, *hcpI*, *vgrG* and *yplA* potentially involved in this process. These three genetic determinant, also shared by *Xenorhabdus*, have been replaced by a selective marker (Km^R or *mCherry*). The knockout *Y. pseudotuberculosis* mutants can be tracked in the EPN life cycle and checked for their ability to colonize or not EPNs as the wild-type strain does. All three knockout mutants have been complemented. Unfortunately, due to lack of time and requirement to perform more control experiments, no conclusion can be drawn from the observed phenotypes. This work could be published if additional experiments, including controls, are performed in the near future.

In contrast to the two previous chapters, chapter 3 will not be presented as a scientific article. First, the three selected genes and their functions will be introduced. Second, the mutational strategy applied to obtain the knockout mutants and their complementation will be described. Finally, results and phenotypes obtained so far will be discussed with recommendations for the further needed experiments.

Chapter 3 – Study of *Yersinia pseudotuberculosis* genetic determinants involved in *Steinernema* colonization.

1. Introduction

In 2008, Heermann and Fuch published a comparative genome analysis of both *Photorhabdus luminescens* and *Yersinia enterocolitica* (Heermann and Fuchs, 2008). This study enlightened a large set of genetic determinants shared by these two Enterobacteriaceae, and notably involved in the host infection process, persistence within the insect, or in host exploitation. Most of these genes, also present in *Xenorhabdus* and *Y. pseudotuberculosis*, are interesting candidates to study in the context of *Steinernema* colonization. Among them, three genes were selected for knockout. The two first encode structural and functional components of the Type 6 Secretion System (T6SS), namely *hcpI* and *vgrG*. The third encodes a phospholipase A termed *yplA*. More details about these three genes are given hereafter.

The T6SS was discovered in *Vibrio cholerae* by the research team of Mekalanos in 2006 (Pukatzki et al., 2006). The T6SS is widely spread among Gram-negative bacteria. It was first intensively studied for its implication in bacterial pathogenesis (*e.g*. *Salmonella* or *Yersiniae*). Some effectors play an important role in virulence towards eukaryotic cells by modifying their cytoskeleton through actin cross-linking (Pukatzki

et al., 2007). However, the presence of the T6SS in non-pathogenic bacteria or even in symbiont-like *Photorhabdus* and *Xenorhabdus* suggested that T6SS could also be involved in other functions or interactions than pathogenesis (Jani and Cotter, 2010). Inter-bacterial interactions such as bactericidal and antimicrobial activities have been described (MacIntyre et al., 2010) or even intra-bacterial interactions involving self versus nonself discrimination (Wenren et al., 2013). The T6SS certainly gives an advantage for host colonization by enabling pathogen to outcompete the host's commensal bacteria (Kapitein and Mogk, 2013). Interestingly, even if the underlying molecular mechanisms are not yet understood, some studies in *Helicobacter* and *Salmonella* suggest a role of the T6SS in the modulation of virulence, promoting a mutualistic behaviour with the eukaryotic hosts. The T6SS could enable a latent sit-and-wait phase ensuring transmission of the bacteria (Jani and Cotter, 2010). This illustrates how versatile the T6SS can be since it can modulate bacterial relations as well as maintain pathogenic or symbiotic interactions with eukaryotic organisms (see Durand et al., 2014 for review).

The T6SS is part of the Sec-independent family of secretion systems, like the Type 1, 3 and 4. T6SS is structurally and mechanistically analogous to the cell-puncturing device of tailed bacteriophages. It functions as a contractile injection machineries that perforate either eukaryotic or prokaryotic cells for effector delivery. A schematic view of the assembly of the T6SS and the effectors translocation is shown at Figure 17. A baseplate complex is formed by 13 core proteins termed Type Six Secretion (Tss) that bridge the inner and the outer bacterial membranes. A sharp conical structure consisting in a trimer valine-glycine repeat G proteins (VgrG)

associated with a Pro-Ala-Ala-Arg repeat containing protein (PAAR) is recruited to this baseplate complex. This VgrG spike is used to pierce

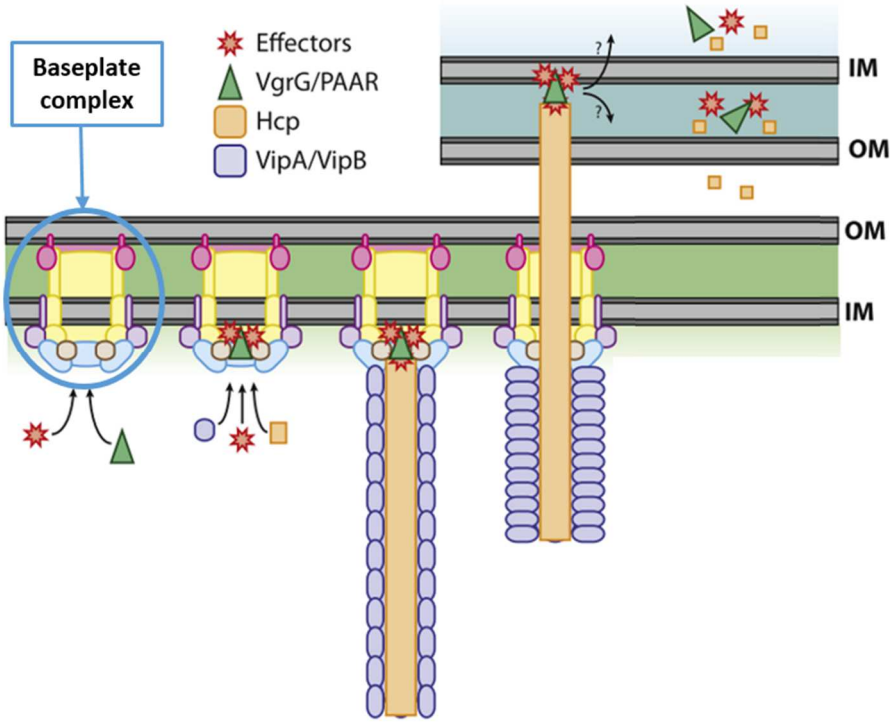

Figure 17: Schematic view of the assembly of the T6SS and the effectors translocation (Adapted from Ho et al., 2013)

A baseplate complex is first formed by 13 Tss proteins. VgrG, PAAR and effector proteins are then recruited to this complex and assemble into the structure. VgrG interaction with PAAR contributes to the overall stability of the T6SS assembly. The VgrG trimer act as a nucleator site for the Hcp tube to polymerize. Then VipA/VipB sheath polymerizes around the growing Hcp tube. Finally a conformation change in the sheath structure results in a contraction event that literaly propels the Hcp tube out of the cell and across a target membrane. Thanks to this contraction the VgrG-effectors are delivered either in the periplasme or in the cytosol of the target cell.

the membrane of the target cells. Using the VgrG trimer as nucleator site, a tail tube is then formed by polymerisation of hexamer rings of hemolysin-coregulated protein (Hcp). A sheath formed by 2 proteins

termed VipA and VipB, polymerizes around the Hcp tail tube. Upon conformational changes, the VipA/VipB sheath contracts and propels the Hcp tail tube that pierces the target membrane thanks to the VgrG spike allowing the delivery of effectors either in the periplasm or in the cytosol of the target cell. Both *vgrG* and *hcp* are essential for functional T6SS (Durand et al., 2014; Ho et al., 2013).

Phospholipases are enzymes that cleave the phospholipids present in the cellular membranes. According to their cleavage site, they are sorted into several classes. Phospholipase A cleaves fatty acid groups from the glycerol backbone of phospholipids. All three pathogenic *Yersiniae* express phospholipases A. One of them, termed YplA (for Yersinia PhosphoLipase A), was first described in *Y. enterocolitica* but homologs are also found in *Y. pseudotuberculosis* and *Y. pestis*. YplA from both *Y. pseudotuberculosis* and *Y. enterocolitica* is involved in mammalian virulence, although its enzymatic activity differs between the two species. First, *Y. enterocolitica* YplA ($YplA_{ent}$) has a greater phospholipase activity than the *Y. pseudotuberculosis* YplA ($YplA_{pst}$). Second, when overexpressed $Ypla_{pst}$ is cytotoxic for *E. coli* while $YplA_{ent}$ overexpression does not affect *E. coli* growth. Despite their high homology, $YplA_{ent}$ and $YplA_{pst}$ may have different enzyme kinetics and/or substrate preferences (Meysick et al., 2009). In *Y. enterocolitica*, YplA is secreted via several Type 3 secretion systems like the flagellar export apparatus (Young and Young, 2002; Young et al., 1999). In fact, $YplA_{ent}$ is part of the flagellar regulon (Schmiel et al., 2000). On the opposite, $YplA_{pst}$ does not belong to the flagellar regulon and is not exported through the flagellar apparatus but is probably exported by another T3SS (Meysick et al., 2009). *Photorhabdus* and *Xenorhabdus* also secrete YplA homologs. Based on this homology,

Heermann and Fuch postulated that *Photorhabdus* and *Xenorhabdus* phospholipases should also play a role in virulence against insects, allowing their survival in the insects. Moreover, *yplA* is expressed at low-temperature suggesting a role in pathogenicity towards insects. Due to its enzymatic activity, YplA might play a role in insects' bioconversion which is essential in the symbiotic lifecycle of *Photorhabdus*/*Xenorhabdus* inside their EPN host (Heermann and Fuchs, 2008).

2. Construction of the mutagenesis cassette delivery vectors

The directed mutagenesis strategy is based on the method used and described in chapter 1. The targeted genes, *hcp1*[2], *vgrG*[3] and *yplA*[4] have been replaced by *mCherry* or by the Kanamycin resistance gene (Km^R) by allelic exchange. The mutagenesis cassettes (MC), termed H-MC, V-MC and Y-MC for *hcp1*, *vgrG* and *yplA* respectively, were generated by combining restriction/ligation reactions and overlap PCR (Shevchuk et al., 2004). MC consists in three fragments: a central fragment (termed CF) containing the reporter gene (*mcherry* or Km^R) flanked by upstream and a downstream fragments (homologous regions termed UF and DF respectively, aimed at replacing the target

[2] YPK_0385 in the annotated genome of *Y. pseudotuberculosis* YPIII (ACA66690.1)

[3] YPK_0401 in the annotated genome of *Y. pseudotuberculosis* YPIII (ACA66706.1)

[4] YPK_1175 in the annotated genome of *Y. pseudotuberculosis* YPIII (ACA67473.1)

gene). First, UF-CF and CF-DF fragments were generated thanks to restriction/ligation reactions. Second, these two fragments were pooled together in an overlap PCR procedure to generate the whole MC. The three MCs were then cloned into the mobilizable suicide vector pKNG101, which confers resistance to streptomycin and carries the counter-selectable marker *sacBR* (Kaniga et al., 1991). The recombinant suicide plasmids were then transferred to *Y. pseudotuberculosis* 4N1 from the conjugative strain *E. coli* SM10λpir. Allelic exchange was conducted in two steps with Streptomycin and sucrose as described in chapter 1. Details about the construction of the three MCs are described hereafter.

2.1. The mCherry mutagenesis cassette

The aim was to replace the *hcpI*, *vgrG* and *yplA* genes by *mCherry*. To do so, a 5'-portion (UF) and a 3'-portion (DF) (about 250 base pairs each) of DNA directly flanking these three genes were PCR amplified from the *Y. pseudotuberculosis* 4N1 strain. CF consists in the strong promotor rrnb P1 from *E. coli*, a ribosome binding site (RBS), the *mcherry* ORF and a L-shaped transcription terminator. The rrnb P1 promotor aims to ensure strong expression of the reporter gene *mcherry* (Zhao et al., 2011). The L-shaped terminator was added to prevent any strong polar effects from the insertion of *mcherry* under the control of rrnb P1. CF was cloned in the pGEM®-T vector (Promega Benelux b.v., Leiden, The Netherlands) and electroporated in One Shot® TOP10 Electrocomp™ *E. coli* (Life Technologies Europe B.V., Ghent, Belgium). The primers designed to amplify UF, DF and CF allowed ligation (*Bgl*II/*Bam*HI) of the UF-CF and CF-DF fragments. The primers used here are listed in Table 7. UF-CF and CF-DF were then

used at 50:50 ratios as DNA templates for a two-step overlap PCR. For the first step no primers were added in the 25µl PCR mix and the following cycling parameters were applied: initial denaturation at 95°C for 3 min followed by 10 amplification cycles (95°C for 20 s; 72°C for 1.5 min). For the second step, 2µl of the first step PCR product together with 2µM of primers amplifying the whole MC were added in a new 30µl PCR mix. The cycling parameters for the second step were the following: initial denaturation at 95°C for 3 min; 25 amplification cycles (95°C for 15 s; 60°C for 30 s; 72°C for 1 min); followed by a final extension at 72°C for 8 min. H-MC, V-MC and Y-MC were cloned in the mobilizable suicide vector pKNG101 (Kaniga et al., 1991) and checked by sequencing with two *mCherry* nested primers BG889 and BG890 (Table 7). The recombinant plasmids were termed pCHMC, pCVMC and pCYMC[5] respectively and were used as *mCherry*-MC delivery vectors. Each of these three plasmids was then transferred into the conjugative strain *E. coli* SM10 λPir. pCHMC, pCVMC and pCYMC were finally conjugated into *Y. pseudotuberculosis* 4N1.

2.2. The Kanamycin cassette

Alternative MCs were generated with the kanamycin resistance gene instead of *mCherry* to allow a more stringent selection (sucrose + Km) of the allelic exchange for *hcpI* and *vgrG* genes. Exactly the same method was used to generate these alternative MCs as the one

[5] pCYMC Plasmid map available in Appendix III

described for the *mCherry* MCs (see section 2.1.). The *Bgl*II restriction fragment corresponding to CF and consisting of the kanamycin resistance gene (without any promoter nor transcription terminator) was cloned in pGEM®-T (Promega Benelux b.v., Leiden, The Netherlands) and electroporated in One Shot® TOP10 Electrocomp™ *E. coli* (Life Technologies Europe B.V., Ghent, Belgium). Primers used for the Km-MCs are listed in Table 7. The Km-MCs mutator plasmids were termed pKHMC and pKVMC[6] and electroporated into the conjugative *E. coli* SM10 λPir which was then conjugated with the *Y. pseudotuberculosis* 4N1.

3. *Yersinia pseudotuberculosis* knockout mutants

Initial integration of the *mCherry*-MC mutator plasmids was selected on specific agar plates containing streptomycin (100 μg ml^{-1}). All initial integrations were PCR checked to determine whether the first recombination occurred with the 5'-portion or the 3'-portion of the *mCherry*-MCs. This PCR allowed also to check if the *mCherry*-MC mutators were integrated in the right locus. The primers used to check *mCherry*-MC vectors integration are listed in Table 8. After purification of the recombinant strains, allelic exchange was selected on agar plates containing sucrose (100 μg ml^{-1}). Allelic exchange was obtained for Y-MC but not for V-MC neither for H-MC which only yielded wild-type

[6] pKHMC and pKVMC plasmid maps available in Appendix III

revertants. The *yplA* knock-out mutant (*yplA* replaced by *mCherry*) was termed *Y. pseudotuberculosis* 4N2 and validated by both PCR and epifluorescence microscopy (Figure 18). The red fluorescence of *Y. pseudotuberculosis* 4N2 was quite weak. After sequencing we figured out that the strong promoter was mutated. Expression of *mCherry* was only under the control of the native promoter of *yplA*.

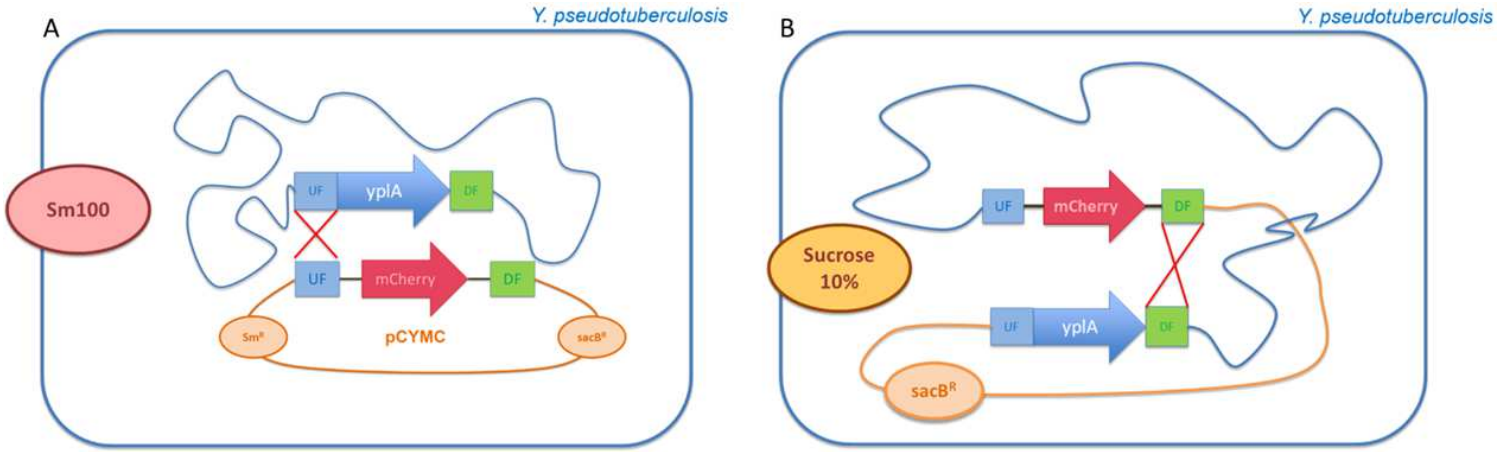

Figure 18: Schematic view of the two recombination steps to replace *yplA* by *mCherry*

A. The suicide plasmid pCYMC is brought to the *Y. pseudotuberculosis* strain by conjugation. The first recombination (here with UF homologous regions symbolised by the red X) occurs under Streptomycin selection (Sm100). The pCYMC integrates into the chromosome.

B. The second recombination is selected by addition of 10% of sucrose in the culture medium. The *sacBR* gene transforms sucrose into a toxic compound for the bacteria. A second recombination step takes place aiming to eliminate the DNA fragment carrying the *sacBR* gene (here with DF homologous regions symbolised by the red X). Eventually the resultant mutant carries the *mCherry* instead of *yplA*.

To overcome the issue encountered with the *mCherry*-MCs, Km-MC mutator plasmids for *hcpI* and *vgrG* were used to obtain knock-out mutants in which *hcpI* and *vgrG* are replaced by Km^R. Initial integrations were as previously described selected with streptomycin (100µg ml^{-1}) and the integration points checked by PCR (Table 8). After purification of the recombinant strains, allelic exchanges were selected on agar plates containing sucrose (100µg ml^{-1}) and kanamycin (30µg ml^{-1}). The *hcpI* and *vgrG* knock-out mutants (*hcpI* and *vgrG* replaced by Km^R) were termed *Y. pseudotuberculosis* 4N3, *Y. pseudotuberculosis* 4N4 and validated by PCR.

4. *Y. pseudotuberculosis* knockout mutant's complementation.

In order to complement the knock-out mutations described above, the broad-host-range plasmid pBBR1MCS has been chosen to bring back the deleted genes and thereby confirm the specificity of the engineered mutations. pBBR1MCS is a mobilisable low copy plasmid already used for stable complementation in *Brucella* sp. and *Yersinia* sp. (Elzer et al., 1995; Marenne et al., 2003; Welch et al., 2011). Moreover, pBBR1MCS carries the Choramphenicol resistance gene (Cm^R). *hcpI* and *yplA* have been successfully cloned into the *Xba*I and *Xho*I sites of pBBR1MCS thanks to primers listed in Table 9. The recombinant plasmids termed pBBR-H and pBBR-Y[7] respectively, were electroporated into the conjugative *E. coli* SM10λpir. pBBR-H and pBBR-Y were checked by sequencing with primers BG1405 and

[7] pBBR-H and pBBR-Y plasmid maps available in Appendix III

BG1406 (Table 9). Unfortunately the *vgrG* ORF could not be cloned into pBBR1MCS due to lack of time.

By conjugation, pBBR-H and pBBR-Y were transferred into *Y. pseudotuberculosis* 4N3 and *Y. pseudotuberculosis* 4N2 respectively. The complemented strains (termed 4N3C and 4N2C) were selected on specific agar plates containing Chloramphenicol (20 μg ml^{-1}). Strains and plasmids used in this chapter are listed in Table 10.

5. Results, Discussion and Perspectives

Y. pseudotuberculosis 4N2 was first tested for EPN's colonization since it was the first knock-out obtained. The method used was the same as the one described in chapter 2. Briefly, 10^6 CFUs of *Y. pseudotuberculosis* 4N2 were injected directly into the haemolymph of *G. mellonella* larvae. As positive control, *G. mellonella* larvae were injected with *Y. pseudotuberculosis* 4N1C. *Steinernema* sp. MW8B were then put on the injected larvae and incubated at room temperature until emergence. Freshly emerged IJs were collected and checked for the presence of *Y. pseudotuberculosis*. The red fluorescence associated with *Y. pseudotuberculosis* 4N2 was so weak that it was impossible to observe the bacteria inside the nematodes. IJs were then crushed on specific agar containing Nal (40 μg ml^{-1}). All the injected G. mellonella died suggesting that the mutants were still virulent. No *Y. pseudotuberculosis* 4N2 could be recovered from the IJs emerged from 6 *G. mellonella* injected larvae. Control IJs were positive in epifluorescence microscopy and *Y. pseudotuberculosis* 4N1C was recovered on specific agar plates containing Nal (40 μg ml^{-1}) and Km (30 μg ml^{-1}). However, this experiment could not be repeated, since positive controls tested after this first attempt failed to yield

productive *G. mellonella* infections. Even if *Y. pseudotuberculosis* 4N2 seems to have lost its ability to colonize EPNs, based on a single experiment, complementation with a cloned *yplA* gene could not be tested for the same reason of positive controls failure.

The other knock-out mutants, *Y. pseudotuberculosis* 4N3 and 4N4 were tested later together with the 4N1C (positive control), the 4N2 (additional experiments) and the complemented strains 4N2C and 4N3C. Each strain was injected into 18 *G. mellonella* larvae. Unfortunately, none of the positive control was successful, either because no emergence occurred or because no *Y. pseudotuberculosis* 4N1C could be recovered from crushed IJs, or observed in epifluorescence microscopy. Actually, no *Yersiniae* have been recovered in any of these second wave of experiments, neither from EPNs nor from insect's haemolymph.

Since it was not in our plans to rear our own *G. mellonella* larvae, these were purchased either in a fishing centre or in a commercial insect production company. From the fourth year of our PhD, we observed a drop in the total IJs emerging from these larvae and sometimes even no emergence at all. We actually know nothing about the rearing conditions of these *G. mellonella* aimed to stay as long as possible in a larval state. It is possible that these larvae are engineered in a way that alters their physiology such as their hormonal balance, their immunity or their microflora. All these parameters could have a significant impact on the ability of EPNs or bacteria (*Xenorhabdus* and *Yersinia*) to infect or even to reproduce and develop properly within these insects. This could be one explanation for the sudden inability of *Y. pseudotuberculosis* 4N1 to colonize or to remain associated with EPNs. Moreover, it becomes problematic to obtain good emergence to

maintain the EPN strains in the lab. Lately the number of IJs emerging from the larvae were smaller than before and the IJs were not only observed after emergence but also males and females indicating a disrupted life cycle within these insects. Therefore, rearing our own *G. mellonella* larvae with controlled microflora or even axenic ones, could solve the inconsistency of these experiments.

Y. pseudotuberculosis 4N2 did exhibit strong red fluorescence as expected. Since YplA is known to be cytotoxic when overexpressed in *E. coli* (Meysick et al., 2009), it is reasonable to state that *yplA* is not strongly expressed in physiological conditions even in *Y. pseudotuberculosis*. Therefore a strong promotor (rrnb P1) was added upstream of *mCherry*. Obviously strong transcription activity in this genomic area was not tolerated by *Y. pseudotuberculosis*. This could explain the difficulties we noticed to obtain allelic exchange when adding sucrose to the medium. Allelic exchange could not be selected neither for *hcpI* nor *vgrG*. *hcpI* or *vgrG* are unlikely to be essential for *Y. pseudotuberculosis* since this is not the case in other bacteria such as pathogenic *E. coli*, *Salmonella* or *Vibrio cholerae*. The kanamycin mutagenesis cassette was used to replace *hcpI* and *vgrG* thanks to a more stringent selection (sucrose + Km). Both allelic exchanges were obtained confirming that neither *hcpI* nor *vgrG* are essential for *Y. pseudotuberculosis*. Again, a strong alteration of the genetic expression could explain why the allelic exchange couldn't be obtained with the *mCherry*-MCs.

Table 7: Primers used to generate the mutagenesis cassettes

Name	Sequence (5' to 3')	Description
BG970	GGGAGATCTTTGGTTGAATGTTGCGCGGTCA GAAAATTATTTTAAATTTCCTCTTGTCAG	*mCherry* CF(1) with *Bgl*II restriction site (underlined), forward primer
BG973	GGGAGATCTAAAAAAAAGGCACGGCTCCAAAA	*mCherry* CF(1) with *Bgl*II restriction site (underlined), reverse primer
BG1268	GGGAGATCTGCATGAGCCATATTCAACGGGA AACGTCTTGC	*Km*^{R} CF(1) with *Bgl*II restriction site (underlined), forward primer
BG1230	GGCAGATCTTTAGAAAAACTCATCGAGCATCA AATGAAACTGC	*Km*^{R} CF(1) with *Bgl*II restriction site (underlined), reverse primer
BG1299	CCCAGATCTGCAAGTGGAACGCGAATTAAAAA CAC	*hcp1* (5'-portion) of H-MC(2) with *Bgl*II restriction site (underlined), forward primer
BG1300	CCCGGATCCTTAACGCCACAAAATAATCTCCG TAGTTAAATACCC	*hcp1* (5'-portion) of H-MC(2) with *Bam*HI restriction site (underlined), reverse primer
BG1065	GGGGGATCCGGCAGCATTTCACCAGAGGC	*hcp1* (3'-portion) of H-MC(2) with *Bam*HI restriction site (underlined), forward primer
BG1066	GGGAGATCTGCCAACTCCAGCTTATTATCTCT ATC	*hcp1* (3'-portion) of H-MC(2) with *Bgl*II restriction site (underlined), reverse primer
BG980	GGGAGATCTCGAACAGGAACGTTCTCGGGGC	*vgrG* (5'-portion) of V-MC(2) with *Bgl*II restriction site (underlined), forward primer

BG981	GGGGGATCCTTAACGGGCAGAGTTCGTCTGC	*vgrG* (5'-portion) of V-MC(2) with *Bam*HI restriction site (underlined), reverse primer
BG978	GCGGGATCCGAGGAGTAGCAGGGATGCCAGA	*vgrG* (3'-portion) of V-MC(2) with *Bam*HI restriction site (underlined), forward primer
BG979	CCCAGATCTTTCTCCGGGGATGTGTTGCGCC	*vgrG* (3'-portion) of V-MC(2) with *Bgl*II restriction site (underlined), reverse primer
BG994	GGGAGATCTCCAACCAGTGAGTTGGGCGA	yplA (5'-portion) of Y-MC(2) with *Bgl*II restriction site (underlined), forward primer
BG995	GGGGGATCCTTACAAACTTTCAGTGAAAGTCC GGG	yplA (5'-portion) of Y-MC(2) with *Bam*HI restriction site (underlined), reverse primer
BG996	GGGGGATCCATGTTTGAACATAAAGGTGAGAT GTCCACGGCG	yplA (3'-portion) of Y-MC(2) with *Bam*HI restriction site (underlined), forward primer
BG997	GGGAGATCTAGGCTGGTTGGCTGTTGGTTAAA GATCGC	yplA (3'-portion) of Y-MC(2) with *Bgl*II restriction site (underlined), reverse primer
BG889	GGCGGCGTGGTGACCGTGACCC	*mCherry* nested forward primer for MC(2) sequencing
BG890	GGGGAAGTTGGTGCCGCGCAGC	*mCherry* nested reverse primer for MC(2) sequencing

(1) Central Fragment containing either *mCherry* or Km^R ORF (see section 2).

(2) Mutagenesis cassette (*mCherry* or Km^R) for H: *hcp1*; V: *vgrG*; Y: *yplA* (see section 2).

Table 8: Primers used to check the integration of the MC delivery vectors

Name	Sequence (5' to 3')	Description
BG1051	GGCCATGTTATCCTCCTCGCCCTTGCTCAAC	*mCherry* (5'-portion), primer reverse
BG1052	CCACCGGCGGCATGGACGAGCTGTAC	*mCherry* (3'-portion), primer forward
BG1053	GCGTATCGGCCTGGCCGGTACCGACA	Upstream V-MC(1) insertion point, primer forward
BG1054	GCGACCTGTCTCTGCCACACCCTTCGGG	Downstream V-MC(1) insertion point, primer reverse
BG1055	GCGGAACTGCGCTGTGTTTGCGCGA	Upstream Y-MC(1) insertion point, primer forward
BG1056	GCGGTATGCCATGCCGTGTCGCCTTGT	Downstream Y-MC(1) insertion point, primer reverse
BG1301	CCTGTAGGAAATTTCCTTAAATTTGCGATGACTTTTCC	Upstream H-MC(1) insertion point, primer forward
BG1302	GCTCTTGTTTGCCTTCAATCGAGGTGCGGTACCAC	Downstream H-MC(1) insertion point, primer reverse
BG1320	GCACCTGATTGCCCGACATTATCGCGAGCCCAT	*KmR* (5'-portion), primer reverse
BG1321	GCCATTCTCACCGGATTCAGTCGTCACTCATGG	*KmR* (3'-portion), primer forward

(1) Mutagenesis cassette (*mCherry* or *Km*^{R}) for H: *hcp1*; V: *vgrG*; Y: *yplA* (see section 2)

Table 9: Primers used to generate complementation vectors

Name	Sequence (5' to 3')	Description
BG1399	GGGCTCGAGATGTTTGCTCATGATAAAGCTAAT	*vgrG* ORF amplification forward primer
BG1400	GGGTCTAGACTACTCCTCTGGATTAAGATCGATC	*vgrG* ORF amplification reverse primer
BG1401	GGCCTCGAGATGAGTGCATCTGTCAGTTTGACTACGCC	*yplA* ORF amplification forward primer
BG1402	CGCTCTAGATGCTCATCCCCCGAAACCCAATAGC	*yplA* ORF amplification reverse primer
BG1403	GGGTCTAGATTACGCTTCGATCGGCGCACGCCAG	*hcpI* ORF amplification forward primer
BG1404	GGGCTCGAGATGCCAACTCCAGCTTATATCTCTA	*hcpI* ORF amplification reverse primer
BG1405	GTAAAACGACGGCCAG	M13 universal forward primer
BG1406	CAGGAAACAGCTATGAC	M13 universal reverse primer

Table 10: List of bacterial strains and plasmids used in this chapter

Strains and Plasmids	Origin (Reference)	Description
pGEM®-T	Promega Corporation	Cloning vector linearized with T-Overhangs for Easy PCR Cloning, *lacZ*α, Ap^R
pGEMt-1	This work	pGEMt carrying the CF for mCherry-MC(1)
pGEMt-2	This work	pGEMt carrying the CF for Km-MC(1)
pKNG101	(Kaniga et al., 1991)	Mobilizable suicide vector, *sacBR*$^+$, *mobRK2*, *oriR6K*, Sm^R
pCHMC	This work	pKNG101 carrying the mCherry-MC(1) for *hcpI*, mcherry-MC(1) delivery vector
pCVMC	This work	pKNG101 carrying the mCherry-MC(1) for *vgrG*, mcherry-MC(1) delivery vector
pCYMC	This work	pKNG101 carrying the mCherry-MC(1) for *yplA*, mcherry-MC(1) delivery vector
pKHMC	This work	pKNG101 carrying the Km-MC(1) for *hcpI*, Km-MC(1) delivery vector
pKVMC	This work	pKNG101 carrying the Km-MC(1) for *vgrG*, Km-MC(1) delivery vector
pBBR1MCS	(Kovach et al., 1994)	Mobilizable complementation vector, *lacZ*α, *mobRK2*, Cm^R
pBBR-H	This work	pBBR1MCS carrying the *hcpI* ORF, complementation vector

pBBR-Y	This work	pBBR1MCS carrying the *yplA* ORF, complementation vector
One Shot® TOP10 Electrocomp™ *E. coli*	Invitrogen™	Electrocompetent strain, *hsd*R, *lac*ZΔM15, *rec*A1, *end*A1
E. coli TOP11	This work	One Shot® TOP10 Electrocomp™ *E. coli* carrying the pGEMt-1
E. coli TOP12	This work	One Shot® TOP10 Electrocomp™ *E. coli* carrying the pGEMt-2
E. coli SM10λpir	(Miller and Mekalanos, 1988)	λ lysogenic conjugative *E. coli* strain expressing the π protein required for replication of plasmids carrying *oriR6K*; Km^R
E. coli CH	This work	*E. coli* SM10λpir carrying the pCHMC
E. coli CV	This work	*E. coli* SM10λpir carrying the pCVMC
E. coli CY	This work	*E. coli* SM10λpir carrying the pCYMC
E. coli KH	This work	*E. coli* SM10λpir carrying the pKHMC
E. coli KV	This work	*E. coli* SM10λpir carrying the pKVMC
E. coli CYC	This work	*E. coli* SM10λpir carrying the pBBR-Y
E. coli KHC	This work	*E. coli* SM10λpir carrying the pBBR-H
Y. pseudotuberculosis 4N1	(Gengler et al., 2015b)	Field isolate, Nalidixic-acid-resistant mutant
Y. pseudotuberculosis 4N1C	(Gengler et al., 2015b)	Field isolate mutagenized with a *mCherry* inserted in the fimbrial A protein A gene; NalR, KmR

Y. pseudotuberculosis 4N2	This work	*Y. pseudotuberculosis* 4N1 with *yplA* replaced by *mCherry*
Y. pseudotuberculosis 4N3	This work	*Y. pseudotuberculosis* 4N1 with *hcpI* replaced by *Km*^{R}
Y. pseudotuberculosis 4N4	This work	*Y. pseudotuberculosis* 4N1 with *vgrG* replaced by *Km*^{R}
Y. pseudotuberculosis 4N2C	This work	*Y. pseudotuberculosis* 4N2 complemented with pBBR-Y
Y. pseudotuberculosis 4N3C	This work	*Y. pseudotuberculosis* 4N3 complemented with pBBR-H

(1) Mutangenesis cassette (see section 2)

General Discussion and Perspectives

This thesis aimed at studying the long-term persistence of some pathogenic bacteria in the environment; thereby bringing new insights into the prevention strategies of animal and human infections by the given pathogenic bacteria.

The main objective of this work was to investigate the ability of pathogenic *Yersiniae* to use soil micro-invertebrates as biological reservoir to ensure their long-term survival in the environment.

Gastro-intestinal pathogens such as *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* have been found on vegetables and in the soil. Under natural conditions, *Yersinia pestis* has also the ability to persist in the soil. Moreover, genomic clues revealed the potential long-term survival of *Y. pestis* in cadavers of infected hosts (Easterday et al., 2012). However, re-emergence of plague after decades of quiescence in very localized foci remains a mystery. The existence of biological micro-reservoirs for pathogenic bacteria has long been speculated. Soil invertebrates have been particularly suspected to act as intermediary hosts and *Steinernema* entomopathogenic nematodes (EPNs) were here considered in this respect. The question addressed in this thesis was to investigate whether a mammalian pathogen like *Yersinia* taxonomically related to *Xenorhabdus* was able to substitute for or "hijack" the symbiotic relationship associating *Xenorhabdus* and *Steinernema* EPNs.

The main objective was achieved thanks to a laboratory model consisting of *Galleria mellonella* insect larvae, *Steinernema* EPNs, fluorescently labelled *Xenorhabdus* and *Yersinia*. This thesis demonstrated that *Y. pseudotuberculosis* is able not only to colonize

Steinernema EPNs but also to remain associated with EPNs during several infection cycles. Moreover, our experiments showed that the ability of *Y. enterocolitica* to colonize *Steinernema* EPNs was reduced compared to *Y. pseudotuberculosis*. Other enteropathogenic bacteria, like pathogenic *E. coli* or *Salmonella*, revealed to be unable to colonize *Steinernema*. Also, our work suggests that *Y. pestis* is unlikely able to remain associated with *Steinernema* EPNs due to antagonist activities of the *Xenorhabdus* symbiont. In addition, three knockout mutants have been generated for genes potentially involved in the colonization of *Steinernema* by *Y. pseudotuberculosis*. Knockout mutants have been complemented and phenotypical characterizations were initiated with some promising preliminary results. This works fulfilled the expectations by shedding light on an unexpected and underestimated biotic micro-reservoir that could play an important role in environmental survival and dissemination of pathogens like *Y. pseudotuberculosis*.

Our laboratory model, the molecular techniques used here as well as the resulting knowledge and findings will be further discussed. The extrapolation of our model to other pathogenic bacteria to address other biological questions will be considered.

1. A laboratory model to demonstrate the ability of enterobacteria to colonize EPNs.

A laboratory model was set up to investigate the ability of pathogenic *Yersinia* to colonize and remain associated with EPNs for a long-term period. This model consists of *Galleria mellonella* insect larvae, *Steinernema* EPNs with or without their natural *Xenorhabdus* symbiont, and fluorescent-labelled *Yersinia* brought artificially either

in the gut of EPNs or in the haemocoele of the insect larva prior to infection. *Yersinia* injected *G. mellonella* larvae were used to feed *Steinernema* IJs. After emergence from the exhausted cadaver, IJs were checked for the presence of *Yersinia* under fluorescent microscopy. When IJs were colonized by *Yersinia*, they were used to infect *Yersinia* free *G. mellonella* larvae. The new generation of IJs emerging from the naive insects were also checked for *Yersinia* colonization and used to complete another infection cycle on naive *G. mellonella* larvae. This has been repeated as long as *Yersinia* were colonizing emerging IJs. Other pathogenic enterobacteria have also been tested with the model. This model addressed three questions: (1) Are the bacteria able to colonize the nematode? (2) Are the bacteria able to remain associated with the nematode for several infection cycles? (3) Can the bacteria replace *Xenorhabdus* as the EPN's symbiont?

1.1. *Steinernema's foreign colonizer, an obligate Xenorhabdus roommate*

Steinernema nematodes are living in symbiosis with species-specific *Xenorhabdus* bacteria. Although aposymbiotic[8] *Steinernema* EPNs can invade and kill insect larvae, they barely reproduce within the larvae and do not survive for a long time. Moreover, Sicard and co-workers studied the impact on the *Steinernema* fitness by replacing its natural *Xenorhabdus* symbiont with another bacteria. They found that the nematode fitness was more impaired when the bacteria were phylogenetically far from the natural symbiont, thus enlighting a

[8] Without natural bacterial symbiont

positive correlation between the phylogenetic distance to the native *Xenorhabdus* symbiont and the impact on *Steinernema* fitness (Sicard et al., 2004). *Steinernema* thus owe its environmental survival to its *Xenorhabdus* symbiont. Unless they can replace the native symbiont, other *Steinernema* colonizing bacteria do not have to cause deleterious effect to *Xenorhabdus*. Otherwise EPNs are considered as prey rather than reservoir for bacteria.

In addition, EPN colonizing candidates have to be resistant to *Xenorhabdus* antimicrobials. Therefore a susceptibility test towards *Xenorhabdus* antimicrobials was developed to assess the resistance of candidate *Steinernema* colonizing bacteria. For this susceptibility test, supernatant from a 48h liquid culture of *Xenorhabdus* sp. TZ01 was used to supplement sterile liquid cultures inoculated with the candidate bacteria. Two concentrations of *Xenorhabdus* supernatant were tested, 4% and 8% of the total culture volume. While some bacteria were found resistant towards *Xenorhabdus* antimicrobials, others were killed or inhibited. Taken together, these tests allowed to compare different bacteria regarding their ability to resist and multiply in the presence of *Xenorhabdus* antimicrobials. The concentration of these antimicrobials in the insect larva upon EPNs infection are probably lower than the ones tested in our study. However, it is possible that these antimicrobials accumulate over time and reach comparable levels after the insect death.

Among the three pathogenic *Yersinia* tested in this work, only *Y. pseudotuberculosis* was able to grow almost normally in the presence of *Xenorhabdus* antimicrobials, while *Y. enterocolitica* and *Y. pestis* were drastically affected. Even if the *Y. enterocolitica* and *Y. pestis* strains tested here have the capacity to colonize *Steinernema* nematodes, *Y.*

pseudotuberculosis is the only one to grow simultaneously with *Xenorhabdus*. It is important to note that a few *Yersinia* strains were tested here with only one *Xenorhabdus* strain. Considering the huge diversity of antimicrobials produced by different *Xenorhabdus* strains, we cannot predict that *Y. pseudotuberculosis* will exhibit the same ability to colonize other *Steinernema*, or that *Y. enterocolitica* or *Y. pestis* will not be able to colonize other *Steinernema*. Analogically to the species-specificity that links *Xenorhabdus* to *Steinernema*, we can assume that some *Yersinia* would colonize more easily some *Steinernema* rather than others. Interestingly two other bacteria, *Serratia marcescens* and *Ochrobactrum* sp., were found to be fully resistant to *Xenorhabdus* antimicrobials. Actually these two bacteria have been isolated directly in the lab from *Steinernema* sp. MW8B nematodes while looking for the natural *Xenorhabdus* symbiont. However, in our experience, neither *Serratia* nor *Ochrobactrum* could efficiently colonize *Steinernema* EPNs as *Y. pseudotuberculosis* does. Dixenic associations between *Photorhabdus* and *Ochrobactrum* in *Heterorhabditis* EPNs have been reported (Babic et al., 2000). *Serratia nematodiphila* has been described as a symbiont for *Heterorhabditidoides chongmingensis* EPN (Zhang et al., 2009). Moreover, *Serratia marcescens* has been isolated several times from entomopathogenic nematodes (Gouge and Snyder, 2006; Tambong, 2013). According to these reports, *Serratia* and *Ochrobactrum* are thus able to colonize at least some *Steinernema* nematodes confirming the correlation between the resistance to *Xenorhabdus* antimicrobials and the ability to colonize *Steinernema*. Vero-toxigenic *E. coli* and *Salmonella* Typhimurium (one field strain each) were tested by us as well and found to be sensitive to *Xenorhabdus* antimicrobials. They could not be recovered from IJs after the first infection cycle. Identifying the antimicrobials secreted by *Xenorhabdus* sp. TZ01 would

help to understand how the bacteria tested here are inhibited and/or how they resist to these antimicrobials. It is unlikely that new families of antimicrobials would come out since *X.* sp. TZ01 is very close to the already described *X. griffiniae* (Appendix II).

1.2. Hosting in Steinernema, a renewable lease

Our laboratory model allowed us to determine if the colonization of *Steinernema* nematodes by *Yersinia* occured for a long period. For the first infection cycle, *Yersinia* was brought artificially by direct injection in the insect haemocoele. Afterwards, *Yersinia* cells found in the new insect larvae were carried over by a *Steinernema* carrier, the latter serving as temporary vector for insect-to-insect transmission of *Yersinia*. A theoretical counts assuming a linear *Y. pseudotuberculosis* dilution all over the consecutive infection cycles showed that no *Y. pseudotuberculosis* CFU can be recovered from EPNs after two infection cycles if no active multiplication occurs. As a result, our experiments demonstrated a multiplication of *Y. pseudotuberculosis* during the EPNs life cycle.

Thanks to the TAIL-PCR methods (further discussed later), we mapped the insertion point of the mini-Tn5 tag in the fimbrial A protein gene of *Y. pseudotuberculosis*. Fimbriae are known to be involved in attachment to eukaryotic cells. This gene is found in two intact copies in the *Y. pseudotuberculosis* chromosome. As far as we can tell, insertion of the transposon did not alter the ability of *Y. pseudotuberculosis* to colonize EPNs nor any of the characters studied herewith (except for the resulting fluorescent phenotype) as compared to the wild-type strain. Nevertheless it cannot be excluded that

Steinernema colonization by the wild-type *Y. pseudotuberculosis* strain would be even more efficient.

In two experiments, IJs_1 carrying *Y. pseudotuberculosis* were unable to complete their life cycle in new insect larvae. Two hypotheses may explain this observation. First it may be that a large *Y. pseudotuberculosis* inoculum generated toxicity towards *Steinernema*. It has been shown that *C. elegans* nematodes fed on a *Y. pestis* lawn for a minimum of 24h showed impaired fitness (Styer et al., 2005). However, *C. elegans* bacterial uptake in Styer's experiments was probably much higher than the 10^3 *Y. pseudotub*erculosis CFUs recovered per *Steinernema* IJ in our model, from the first emergence and onwards. In addition, *Steinernema* EPNs graze and complete their reproductive cycle on *Y. pseudotuberculosis* lawns grown on Petri plates. This is true for both EPNs carrying their natural *Xenorhabdus* symbiont and EPNs cured of their symbiont. The hypothesis of a possible toxic effect of *Y. pseudotuberculosis* on *Steinernema* is therefore not plausible. Alternatively, since the *G. mellonella* larvae used in our experiments were not reared in the lab but bought from insect commercial suppliers (fishing shops), it may be that these larvae were reared in a way that delayed pupation or were treated to resist microbial infections, or both. Besides the two IJs_1 failed infections, we also observed consistently that the *Steinernema* individuals not infected by *Y. pseudotuberculosis* also failed to initiate or complete a successful infection of the insect larvae. This may also explain the low number of IJs emerged after 7 infection cycles. Rearing our own G. *mellonella* larvae and controlling their commensal microflora could resolve this infectivity problem, thereby increasing the reproducibility of our experimental model. In addition, laboratory practices suggest that alternating the insect food sources for EPNs allow to longer maintain their infectivity and their ability to

multiply within insects. Moreover, the Tanzanian *Steinernema* isolate (MW8B), have been sent to other labs producing their own *G. mellonella* larvae. The MW8B isolate fail to reproduce after some time in these labs as well, suggesting that *G. mellonella* is probably not the best insect larva to feed this *Steinernema* isolate. The use of more "natural food sources" such as insect larvae living in the soil conversely to *G. mellonella*, might solve the EPN multiplication issue encountered here.

Despite the fact that *Y. enterocolitica* is more sensitive towards *Xenorhabdus* antimicrobials, IJs failed in many attempts to mature in *G. mellonella* larvae injected with *Y. enterocolitica*. In addition, IJs1 carrying *Y. enterocolitica* failed to complete their cycle in a new naive *G. mellonella* larva. The pathogenicity of *Y. enterocolitica* towards *C. elegans* nematodes has been demonstrated by Spanier et al. (2010) who conducted a *C. elegans* oral infection with low bacterial doses of 10^4 *Y. enterocolitica* CFUs per worm and showed that *Y. enterocolitica* concentration reached up to 10^6 CFUs per worm four days and later causing nematode's death (Spanier et al., 2010). The actual oral toxicity of *Y. enterocolitica* towards EPNs should therefore be investigated in our model, and could explain the observed lower efficiency of *Y. enterocolitica* infections. It was also shown that, at room temperature or at 37°C, injected 10^5 CFUs of *Y. enterocolitica* kills *G. mellonella* larvae within 72h. Also, 10^6 CFUs of *Y. pseudotuberculosis* or *Y. pestis* are needed to kill 50% of the larvae within the same time frame at 37°C. However, at room temperature, 85% of the *Y. pseudotuberculosis*- or *Y. pestis*-injected larvae survived (Erickson et al., 2011). Therefore injection doses lower than 10^6 CFUs of *Y. enterocolitica* in the insect larvae could improve EPNs development in the injected insects in our model.

Steinernema infection experiments were conducted with *Serratia marcescens* but the EPNs failed to complete their cycle in injected larva. Some *Serratia* (like *S. entomophila* or some strains of *S. marcescens*) are more entomopathogenic than *Y. pseudotuberculosis* and use the type 6 secretion system to target and kill bacterial competitors (Murdoch et al., 2011). This suggests that the 10^6 CFUs starting inoculum we used might have been excessive and allowed *Serratia* to dominate the microflora feeding on the insect macromolecules. This may have impaired *Xenorhabdus* multiplication and impaired subsequent EPNs development. From an environmental perspective, these observations suggest that *Serratia* as well as *Y. enterocolitica* may accidentally reach the gut of *Steinernema* but are unlikely to colonize and multiply within the EPN as *Y. pseudotuberculosis* does. Both *Y. pseudotuberculosis* and Xenorhabdus genomes do host a T6SS gene set but these systems – if expressed at all – are not detrimental to the other bacterium since both *Y. pseudotuberculosis* and *Xenorhabdus* can co-exist within the nematode as demonstrated in chapters 1 and 2.

The finding that *Ochrobactrum* can colonize *Steinernema* EPNs is more surprising than the case of *Serratia*. *Ochrobactrum* is not known to be an insect pathogen and belongs to the *Brucellaceae* family. Since the last decade, there is more and more concern about *Ochrobactrum* shifting from an environmental plant growth-promoting bacterium towards an opportunistic human pathogen. *Ochrobactrum* bacteria are resistant to multiple antimicrobials as confirmed by the present work, and are phylogenetically very close to the anthropozoonotic pathogens *Brucella* spp. (Scholz et al., 2008). Colonization of micro-invertebrates such EPNs by *Ochrobactrum* could bring new insights in our understanding of the emergence of pathogenic life-style among *Brucellaceae* species (Aujoulat et al., 2014).

1.3. An insect-to-insect transmission model, a light on future work

Keeping in mind that the scope of this work was not to faithfully mimic what could happen in nature but rather to demonstrate the validity of a laboratory transmission model, two European strains of *Y. pseudotuberculosis*, selected on the basis of their resistance towards *Xenorhabdus* antimicrobials, have been tested with an African EPN strain. This thesis is innovative in the study of long-term persistence of human pathogenic bacteria in EPNs. There is no doubt that more work is required for instance with several *Yersinia* and *Steinernema* strains to really generalize the observation made herein. In addition, repeating the same experiments with *Heterorhabditis* EPNs could further confirm EPNs as biotic reservoir for *Yersiniae*. In *Heterorhabditis* EPNs, *Yersinia* would be in close contact with the *Photorhabdus* symbiont not only in the insect cadaver but also in the IJs gut. Although no receptacle is present in *Heterorhabditis*, a strict selection occurs to keep the natural *Photorhabdus* symbiont which also possesses a lot of genomic regions involved in the specificity of nematode host interaction (Gaudriault et al., 2006). Although a larger diversity of non-symbiotic bacteria can be found together with *Xenorhabdus* within the intestine of *Steinernema* species, it has been established in several *Heterorhabditis* species that *Photorhabdus* is present among other bacteria within the digestive tract (Babic et al., 2000; Boemare et al., 1996; Jackson et al., 1995). *Heterorhabditis* gut colonization might be more competitive as compared to *Steinernenma*'s gut, hence *Y. pseudotuberculosis* could multiply as much as it does within *Steinernema*'s gut. Although *Ochrobactrum* can co-exist with *Photorhabdus* in *Heterorhabditis*, it was not clearly established if *Ochrobactrum* can maintain during several infection cycles within the

nematode. No distinction has been done between *Ochrobactrum* already present in *G. mellonella* larvae and *Ochrobactrum* carried by EPNs. The same question remains open for *Serratia*. Here also, *G. mellonella* reared in conditions involving microflora control (possibly axenic growth) could help resolve this lingering issue.

The knowledge brought by our experimental model so far supports the hypothesis of EPN as biotic reservoir for *Y. pseudotuberculosis* in the environment. The finding of *Y. pseudotuberculosis* in EPNs recovered from natural soil samples would definitely demonstrate the validity of our hypothesis. We anticipate that the required field work we could not perform in the framework of this thesis, is now warranted and far more justified scientifically and economically.

1.4. Micro-Invertebrates as biological reservoir for human pathogenic bacteria

Other micro-invertebrates have been studied and are still studying regarding their ability to act as biological reservoir for human and mamal pathogenic bacteria. One in particular, the free-living amoebae (FLA), raised the scientific community interest mainly for the past two decades. FLAs are ubiquitous protozoa widely distributed in water, soil and atmosphere environments (Garcia et al., 2013; Kingston and Warhurst, 1969). Some FLA are studying for their implication into human infections (Visvesvara et al., 2007). However more and more interest is given to the FLA as carrier or reservoir for mamal pathogenic bacteria (Cateau et al., 2014). Some pathogenic bacteria can indeed survive the amoebae phagocytosis and survive within the amoebic cells. The water-born *Legionella pneumophila* has been found associated

with FLA and can actively multiply in these protozoa (Borella et al., 2005). *Pseudomonas aeruginosa* is also a water-born pathogen found inside FLA (Calvo et al., 2013). Moreover, *P. aeruginosa* could have a beneficial effect on the growth and survival of some amoebae suggesting a possible commensalism between these two micro-organisms (Anacarso et al., 2010). In addition it has been shown that amoebae are able to resist chlorination treatment allowing as well the survival of their associated bacteria (King et al., 1988). Amoebae thus offer protection to pathogenic bacteria strengthening the commensalism suggested by Anacarso and co-workers. The survival of the well-known water-born pathogen Vibrio cholerae has also been described within amoebae. More interestingly a symbiotic relationship between *V. cholerae* and a FLA has been suggested (Abd et al., 2007).

The role of amoebae in the persistence of pathogenic bacteria have been well studied so far. New researches are lead in order to describe the interactions between bacteria and amoebae. Even if the survival of Yersiniae within amoebae would be really interesting to test, this work focused on another potential micro-invertebrate host for pathogenic bacteria clearly adding new insights in the study of interaction between micro-invertebrates and mamal pathogenic bacteria.

2. Dual fluorescence and confocal microscopy

2.1. An easy way to label bacteria

Bacteria studied in this thesis have been labelled using mini-Tn*5-gfp* derivative. This technique is easy to perform and quickly provides labelled insertion mutants. A screening step is required

though to select clones with appropriate fluorescence. Because of the random character of Mini-Tn*5* transposon insertion, it is necessary to map the insertion point to determine whether the phenotype to be studied could be affected or not by the insertion. We used and enlightened a rapid and powerful method to map the insertion point of our transposon. TAIL-PCR is barely known in molecular bacteriology while broadly used among plant and mammalian studies. This is probably due to the fact that inverse PCR – a technique available for transposon insertion mapping in bacteria – is not at all convenient for genomes larger than 10^9 base pairs. Unlike inverse PCR, TAIL-PCR is simple and efficient in genomic walking studies as it requires no fastidious restriction or ligation steps (Liu and Whittier, 1995). In addition, a molecular tool was developed to swap the fluorescence marker of the mini-Tn*5*-*gfp* labelled bacteria. This tool allowed us to re-engineer the *gfp*-labelled *Y. pseudotuberculosis* while keeping the genetic characteristics identical to the parent strain. Thanks to TAIL-PCR and our swapping method we overcame the time-consuming steps of screening and mapping a new mini-Tn*5* insertion mutants. Based on homologous recombination, our tool could be used to re-engineer any bacterial strains labelled with *gfp* or one of its variants.

2.2. New localization pattern for Xenorhabdus

The *mCherry*-labelled *Y. pseudotuberculosis* allowed dual localization with *gfp*-labelled *Xenorhabdus* by confocal microscopy. While confirming the simultaneous colonization of *Xenorhabdus* and *Y. pseudotuberculosis*, a yet undescribed localization pattern of *Xenorhabdus* was observed. In some males only, the receptacle appeared to be split into several vesicles. Confocal images showed a clear segregation between several vesicles colonized by *Xenorhabdus*

(Appendix IV). This pattern has been observed in three independent samples of *Steinernema* grazed on a *gfp*-labelled *Xenorhabdus* lawn. The number of vesicles varied between 2-3 and 6-8. Segregation of the receptacle might allow the nematode to save some symbiont while reproductive organs develop. An in-depth analysis of the nematodes age and their development is obviously required here to understand why, how and when segregation of the receptacle occurs. Although this phenomenon may be due to an artefact from EPNs artificially cultured on agar plates, a new mechanism of host-interaction between *Steinernema* and *Xenorhabdus* cannot be ruled out.

3. Genetic determinants potentially involved in *Steinernema* colonization

Several knockout mutants have been engineered and complemented in *Y. pseudotuberculosis*. Unfortunately no conclusion could be drawn from these mutants since the positive controls stopped working as discussed in chapter 3. Besides the fact that the provided insect *G. mellonella* larva might have been reared in a way impairing the nematodes development, continuously feeding the EPNs with the same insects may also impaired their multiplication efficiency within *G. mellonella*. The genes knocked-out are *Xenorhabdus* orthologs and have been discussed in chapter 3. Other genes could also be essential for *Yersinia* to colonize *Steinernema*. Heungens et al. (2002) found that *Xenorhabdus* deletion mutants for *aroA* and *serC* that encode amino acid biosynthetic enzymes, are drastically affected in their ability to colonize the receptacle. However, in *Xenorhabdus* these mutants have shown growth, motility and enzymatic defects suggesting that *aroA* and *serC* are not specifically involved in colonization (Heungens et al.,

2002). Nonetheless, *aroA* and *serC* homologs are found in *Yersinia* and could have an impact on *Steinernema* colonization. Other genes coding for transcription factors or regulatory proteins have been shown to be involved in *Steinernema* colonization. Although useful as first screening candidates, regulatory gene knockout only indirectly affect functions essential for the colonization of *Steinernema* or the symbiotic association with them. The genes under their control are probably more interesting. Since *Serratia* and *Ochrobactrum* seem to have the ability to colonize *Steinernema* EPN, a genomic comparison with *Y. pseudotuberculosis* could enlighten new genes potentially involved in nematodes colonization. We already know that a *yplA* ortholog is present in *Serratia* (Heermann and Fuchs, 2008) which, in regard of our preliminary results (chapter 3), may suggest that *yplA* plays a role in *Steinernema* colonization.

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Appendix I: Scientific communication

Publications

Published paper

Gengler, S., Laudisoit, A., Batoko, H., Wattiau, P., 2015. Long-Term Persistence of *Yersinia pseudotuberculosis* in Entomopathogenic Nematodes. PLoS One 10(1), e0116818.

Gengler, S., Batoko, H. and Wattiau, P., 2015. Method for fluorescent marker swapping and its application in *Steinernema* nematode colonization studies. Journal of Microbiological Methods 113, pp. 34-37

In preparation papers

1. Tailliez, P., Pagès, S., Ginibre, N., Ogier, J.-C., **Gengler, S.**, Haukeland, S., Wattiau, P., Laudisoit, A. Description of Xenorhabdus pwaniensis sp. nov., the symbiotic bacterium associated with a new Steinernema species isolated in Tanzania. *In preparation*.
2. Haukeland, S., Kora-Elborai, Malan, A., Mwaitulo, S., Laudisoit, A., **Gengler, S.**, Tailliez, P. F., S. Hauser, S., Kanga, B., Kimenju, J., Manrakhan, A., Samuel, A., and Coyne, D. A first review on the status of Entomopathogenic Nematodes (Steinernematidae, Heterorhabditidae) in Africa. *In preparation*.

Oral presentations

1. **Gengler, S.**, Laudisoit, A. and Wattiau, P., Entomopathogenic nematodes as disseminating agent for *Yersinia pseudotuberculosis*: A Matryoshka doll model. International Congress on Invertebrate Pathology and Microbial Control

45th Annual Meeting of the Society for Invertebrate Pathology August 5-9, 2012, Buenos Aires, Argentina.

2. **Gengler, S.**, Laudisoit, A. and Wattiau, P., Entomopathogenic nematodes as disseminating agent for *Yersinia pseudotuberculosis*: A laboratory model. 31st International Symposium of European Society of Nematologists, September 23-27, 2012, Adana, Turkey. (ESN Grant)
3. **Gengler, S.**, Laudisoit, A. and Wattiau, P., *Steinernema-Xenorhabdus* symbiosis and *Yersinia pseudotuberculosis*: Brothers in arm or hacking system? 6th International Congress of Nematology, May 5-9, 2014, Cap Town, South Africa.

Poster presentations

1. Laudisoit, A., **Gengler, S.**, Haukeland, S., Mast, J., Wattiau, P. Yersinia pseudotuberculosis can intrude the parasitic life cycle of entomopathogenic nematodes. 13th annual conference of the Belgian Society of Microbiology, November 18-19, 2010, Brussels, Belgium. (Second best poster award)
2. **Gengler, S.**, Laudisoit, A. and Wattiau, P. Entomopathogenic nematodes as disseminating agent for *Yersinia pseudotuberculosis*: a laboratory model. 4th symposium of the Belgian Wildlife Disease Society, October 7, 2011, Brussels, Belgium. (Second best poster award)
3. **Gengler, S.**, Laudisoit, A. and Wattiau, P. Entomopathogenic nematodes as disseminating agent for *Yersinia pseudotuberculosis*. 14th annual conference of the Belgian Society of Microbiology, November 16, 2011, Brussels, Belgium.

Appendix II: Description of *Xenorhabdus pwaniensis* sp. nov., the symbiotic bacterium associated with a new *Steinernema* species isolated in Tanzania

This work has been submitted as a taxonomic note but required the description of the associated Tanzanian *Steinernema* host to be published. The *Steinernema* description is ongoing in the Dr. Vladimir Puza lab (Institute of Entomology, Czech Republic).

Description of *Xenorhabdus pwaniensis* sp. nov., the symbiotic bacterium associated with a new *Steinernema* species isolated in Tanzania

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1. Summary

The symbiotic bacterium (TZ01^T) was isolated from a new species of insect-pathogenic nematode of the genus *Steinernema*. On the basis of 16S rRNA gene sequence similarity, this bacterial isolate was shown to belong to the genus *Xenorhabdus* in agreement with the genus of its nematode-host. The accurate phylogenetic position of this new isolate was defined using a multigene approach and showed that isolate TZ01^T shares a common ancestor with *X. griffiniae* ID10^T and *X. ehlersii* DSMZ16337^T, the symbiotic bacteria associated with *Steinernema hermaphroditum* and *Steinernema longicaudum*, respectively. The nucleotide identity (less than 96.3%) between TZ01^T, *X. griffiniae* ID10^T and *X. ehlersii* DSMZ16337^T calculated on the concatenated sequences of five gene fragments encompassing 4294 nucleotides, several phenotypic characters and fatty acids profiles between these three bacterial species allowed genetic and phenotypic differentiation of isolate TZ01^T from its two closely related species. TZ01 ^{T} was shown to be highly pathogenic for *Galleria mellonella* (Linnaeus, 1758) and *Spodotera littoralis* (Boisduval, 1833). TZ01^T therefore represents a new species of entomopathogenic bacteria, for which the name *Xenorhabdus pwaniensis* sp. nov. is proposed, with the type strain DSM25309^T (=CIP110340^T).

2. Introduction

Bacteria of the genus *Xenorhabdus* (Thomas & Poinar, 1979) are symbiotically associated with insect-pathogenic nematodes of the genus *Steinernema* (Travassos, 1927). The bacterial symbionts have been shown to be highly pathogenic for insects and to contribute

efficiently to the development of their nematode host during the parasitism of insects. Until now, 22 *Xenorhabdus* species have been described (Akhurst & Boemare, 1988; Nishimura *et al*., 1994; Lengyel *et al*., 2005; Somvanshi *et al*., 2006; Tailliez *et al*., 2006, 2010 and 2011), some of them have been shown to be associated with several *Steinernema* species (Tailliez *et al*., 2010). Amongst the 70 *Steinernema* species described to date, five of them were isolated from the African continent such as *Steinernema karii* from Kenya (Waturu *et al*., 1997), *Steinernema yirgalemense* (Nguyen *et al*., 2004) and a new *Steinernema* species (*S. ethiopiense* sp. n., Tamiru *et al*., unpublished) from Ethiopia, *Steinernema citrae* (Malan *et al*., 2011) and *Steinernema khoisanae* (Nguyen *et al*., 2006) from South Africa. *S. karii* was found to be associated with *Xenorhabdus hominickii* (Tailliez *et al*., 2006) while for *S. yirgalemense*, "*S. ethiopiense* sp. n." and *S. citrae*, their bacterial symbionts are not yet characterized. *S. khoisanae* was found to be associated with an undescribed *Xenorhabdus* species which isolate was named SF87 (accession n° HQ142625 for the 16S rRNA gene sequence). We described herein the symbiotic bacteria of the genus *Xenorhabdus* associated with a new undescribed *Steinernema* species isolated in Tanzania (Mwaitulo *et al*., 2011). This new species is phylogenetically related to *Steinernema karii* and "*Steinernema ethiopiense*".

3. Material & Methods

The nematode was extracted from soil by the *Galleria* baiting method (Bedding & Akhurst, 1975) in the region of Pwani situated along the coast, in Eastern Tanzania. Nematode genomic DNA was extracted from infective juveniles (IJs). IJs were placed in Eppendorf tubes at -80 °C for 30 min and then incubated at 65 °C for 15 min. The

pellets of IJs were then collected after centrifugation (13 000 g for 10 min), resuspended in 180 µl of lysis buffer included in the NucleoSpin®Tissue kit (Macherey-Nagel, Germany) supplemented with 25 µl of proteinase K (23 µg μl^{-1}) and incubated at 56 °C for at least 3 h to achieve cell lysis. DNA purification was then performed in accordance to the manufacturer's recommendations. A ~850-bps fragment corresponding to the Internal Transcribed Spacer region (ITS) of the ribosomal DNA was amplified in a 50-µl reaction solution containing the *Taq* polymerase according to the manufacturer's protocol (Invitrogen, France). The amplification primers were ITS-forw: 5'-GGACTGAGCTGTTTCGAGA-3' targeted the 3'-terminus of the small subunit (SSU) rDNA and ITS-rev: 5'-TACTGATATGCTTAAGTTCAGCG-3' targeted the 5'-terminus of the large subunit (LSU) rDNA. PCR was carried out in a Bio-Rad thermocycler (Bio-Rad, France) programmed for 30 cycles of amplification: after an initial 4 min denaturation step at 94°C, each cycle consisted of 30 s at 94 °C, 30 s at 52 °C, and 90 s at 72 °C, followed by a last step at 72 °C for 5 min. Amplification of the LSU rDNA that included the D2 and D3 domains was performed as described previously (Stock *et al*., 2001). PCR fragments were controlled by electrophoresis in agarose gel (1 % agarose in 1X TAE buffer) to confirm size and yield. DNA fragments were then purified using a high purity purification kit (Roche Diagnostic, France) and sequenced by the sequencing service of MWG (Eurofins MWG Operon, Germany) using the primers listed above and those described by Stock *et al*. (2001) as sequencing primers. *Xenorhabdus* cells were obtained from the infective stages of the nematode by the hanging-drop technique (Poinar, 1966) and isolated by plating on nutrient agar supplemented with 0.004 % (w/v) triphenyltetrazolium chloride and 0.0025 % (w/v)

bromothymol blue (NBTA medium) at 28°C (Akhurst, 1980) for 48h. The bacterial isolate named TZ01 was examined for the main phenotypic characteristics of the genus *Xenorhabdus* using the methods of Boemare & Akhurst (1988) and was stored at -80 °C in LB broth containing 15 % glycerol (v/v). Fatty acid analyses were carried out by the Identification Service of the DSMZ (Braunschweig, Germany) using an Agilent 6890N gas chromatograph and version 6.1 of the MIDI Inc Sherlock MIS software. The method of preparation of the samples is the standard method described in MIDI "Technical Note 101" (http://www.midi-inc.com/pdf/MIS_Technote_101.pdf). Bacteria were grown on trypticase soy broth agar (TSBA) at 28 °C for 24 h. The fatty acid data were analyzed using Partial Least Square Discriminate Analysis (PLS-DA) included in the SIMCA-P software, version 10.0.4 (Umetrics AB, Umea, Sweden) where the percentages of each fatty acid were considered as variables X and the classification of each isolate in one species was considered as variable Y, so that PLS-DA finds the relationship which characterized the composition of fatty acids of isolates belonging to a determined species compared to the others. Three independent replicates were obtained for TZ01^T and ID10^T, and only one replicate was obtained for each of the five *X. ehlersii* strains included in this study. Bacterial DNA extraction, gene fragment amplification and sequencing were as previously described (Tailliez *et al*., 2006, 2011). The phylogenetic analysis of isolate TZ01^T was based on a multigene approach (Tailliez *et al*., 2011) including five universally conserved protein-coding sequences (*recA*, *gyrB*, *dnaN*, *gltX* and *infB*) using PAUP software (Swofford, 2003) for distance and parsimony trees and PhyML (Guindon & Gascuel, 2003) for maximum likelihood trees. Phylogenetic trees were calculated on individual gene sequences, on concatenated sequences (5 gene sequences for the

bacteria and LSU fragment + ITS for nematodes) using the "concatenate" function included in the multiplatform graphical user interface Seaview (Gouy *et al*., 2010). The 16S rRNA gene sequences were also used for the classification of the new bacterial isolate and for comparison with previous analysis including representatives of already described (Tailliez *et al*., 2010) and putative new *Xenorhabdus* species (Isolates SF87, Mecklenburg and CR9). The percent nucleotide identity between DNA pair of sequences was obtained using the FASTA package (Pearson, 1990). Sawyer's test for detecting recombination intervals based on the detection of shared patterns of polymorphisms (Sawyer, 1989) was performed with the computer program GENCONV (http://www.math.wustl.edu/~sawyer). Synonymous and non-synonymous substitutions between pair of sequences was calculated using the codon-based test and the Fisher's exact test of neutrality implemented in Mega 5.05 (Tamura *et al*., 2011). *In vivo* pathogenicity assays were performed as previously described (Givaudan & Lanois, 2000; Sicard *et al*., 2006). *Galleria mellonella* (Linnaeus, 1758) was reared in the dark in aired plastic boxes at 28 °C, 65 % relative humidity, on a diet composed of beeswax (36 g kg^{-1}), brewer's yeast (72 g kg^{-1}), honey (180 g kg^{-1}), glycerin (230 g kg^{-1}) and wheat flour (480 g kg^{-1}). The cutworm *Spodoptera littoralis* (Boisduval, 1833) was reared with a photoperiod of 12 h on an artificial diet at 23 °C (Poitout & Bues, 1970). Last instar of the wax moth *G. mellonella* and fourth-instar of the cutworm *S. littoralis* were surface sterilized with 70 % (v/v) ethanol prior to intrahemocoelic injection of *Xenorhabdus* cells or *Escherichia coli* cells for control. Twenty larvae of each insect were injected with 20 µl of bacterial solution containing 10^2 (for *G. mellonella*) and 10^3 (for *S. littoralis*) bacterial cells. Treated larvae were individually incubated for up to 96h and the time to death was recorded.

4. Results

4.1. Phylogenetic position of Steinernema sp., strain S3, nematode host of the symbiotic bacterial isolate TZ01^T.

Several markers (nuclear sequences corresponding to ITS, LSU and small subunit rDNA fragments, and mitochondrial sequences corresponding to cytochrome oxydase I and 12S ribosomal DNA fragments) were used to study the phylogeny of the genus *Steinernema* (Stock *et al*., 2001, Spiridonov *et al*., 2004, Nadler *et al*., 2006). Among them, ITS and LSU rDNA gene sequences are the most widely used (e.g. in a recent new *Steinernema* species description by Qiu *et al*., 2011). Using the LSU and the ITS rDNA sequences available in GenBank, we firstly determined the phylogenetic position of strain S3 within the clade V "*arenarium-glaseri-karii-longicaudum*" (Stock *et al*., 2001; Spiridonov *et al*., 2004). Then, the LSU and the ITS rDNA sequences available for strains belonging to each representative species of this *Steinernema* group were concatenated. Thus, we showed that nematode strain S3 shares a common ancestor with *S. karii* and *S. ethiopiense* (Figure 19) forming a group of phylogenetic related species isolated from Africa.

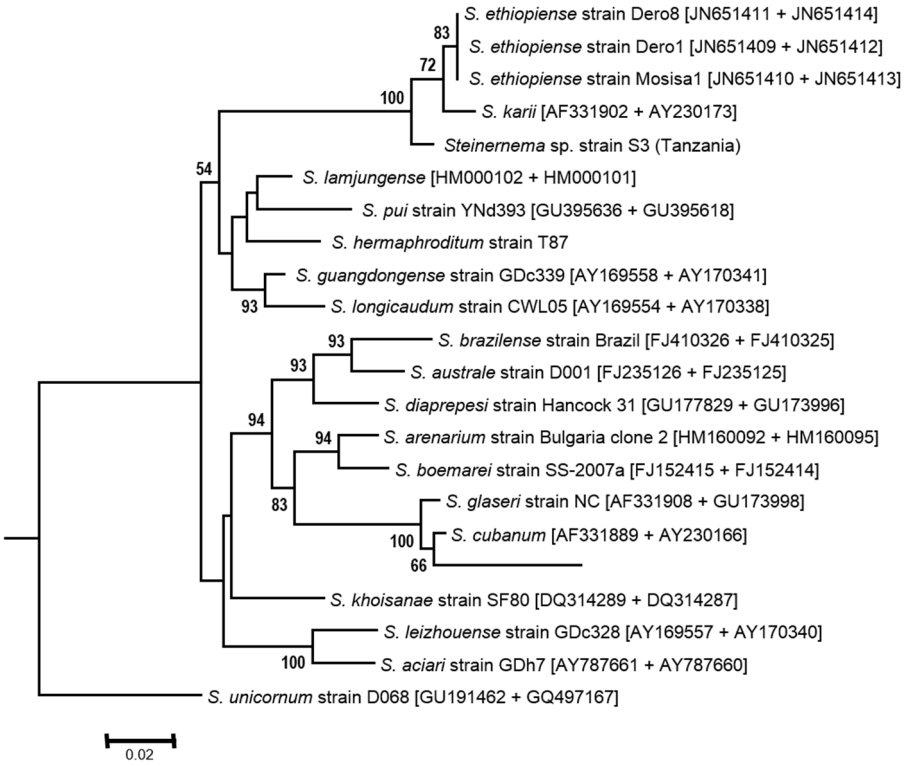

Figure 19: Maximum likelihood phylogenetic tree of the "*arenarium-glaseri-karii-longicaudum*" clade including *Steinernema* sp. strain S3.

The analysis was based on the concatenated LSU gene and ITS rDNA sequences. Sequences were aligned using MUSCLE 3.7 (Edgar, 2004) implemented in the "Phylogeny.fr" plateform (Dereeper *et al*., 2008). Gaps were excluded. Substitution model was GTR with gamma distributed rates and invariant sites. The sequences of *S. unicornum* strain D068 were chosen as closely related outgroup. GenBank accession numbers of the sequences are in brackets. Bar represents 2 % divergence. Bootstrap values (Felsentein, 1988) of more than 50 %) are indicated at the nodes. The group including *Steinernema* strain S3 and two other *Steinernema* species isolated from Africa was highly supported (Bootstrap value = 100%).

4.2. Taxonomic and phylogenetic position of bacterial isolate TZ01^T.

The 16S rDNA distance tree (Figure S1, supplementary data) showed that the sequence of *Xenorhabdus* isolate TZ01^T was highly

similar to those of *X. ehlersii* strains included in our study (98.6 % identity with the sequence of the type-strain DSM16337^T), the bacterial symbiont of *Steinernema longicaudum*. This result confirms that isolate TZ01^T belongs to the genus *Xenorhabdus* in agreement with the genus of its nematode-host. A more accurate analysis based on the five concatenated gene sequences confirmed the phylogenetic position of isolate TZ01^T in a clade including *X. griffiniae* and *X. ehlersii* (Figure 20) whatever the method used (distance, parsimony and ML). The phylogenetic position of isolate TZ01^T sharing a common ancestor with *X. griffiniae*, is identical for three (*dnaN*, *gltX*, *infB*) out of the five genes tested (Figures S4 to S6, supplementary data). The phylogenetic positions of TZ01^T given by the *gyrB* and the *recA* genes were in general agreement with the previous analysis except that TZ01^T was at a basal position in the case of *gyrB* (Figure S3, supplementary data) whereas *X. griffiniae* ID10^T was at the basal position using *recA* (Figure S2, supplementary data). Such a discrepancy was also observed for strain KR02 for which three genes (*gyrB*, *dnaN* and *infB*) suggested that this strain belongs to the species *X. ehlersii* whereas two other genes (*recA* and *gltX*) suggested a different evolutionary history (basal position of the clade). At least, the phylogenetic position of *X. kozodoii* as a closely related species of this clade was confirmed by the five genes except that *recA* proposed a more basal position for this species. The Sawyer's test for recombination applied to our five gene sequences did not show putative recombination events suggesting that recombination was not the main mechanism involved in the evolution of these genes for *Xenorhabdus*. Comparison of non-synonymous (dN) and synonymous substitutions (dS) per site using the codon-based test and the Fisher's exact test of neutrality between pair of sequences (Mega 5.05) showed that the hypothesis of strict neutrality was rejected for the *recA*

sequence of KR02 compared to those of DSM16337^T, CN01, TZ01^T and ID10^T.

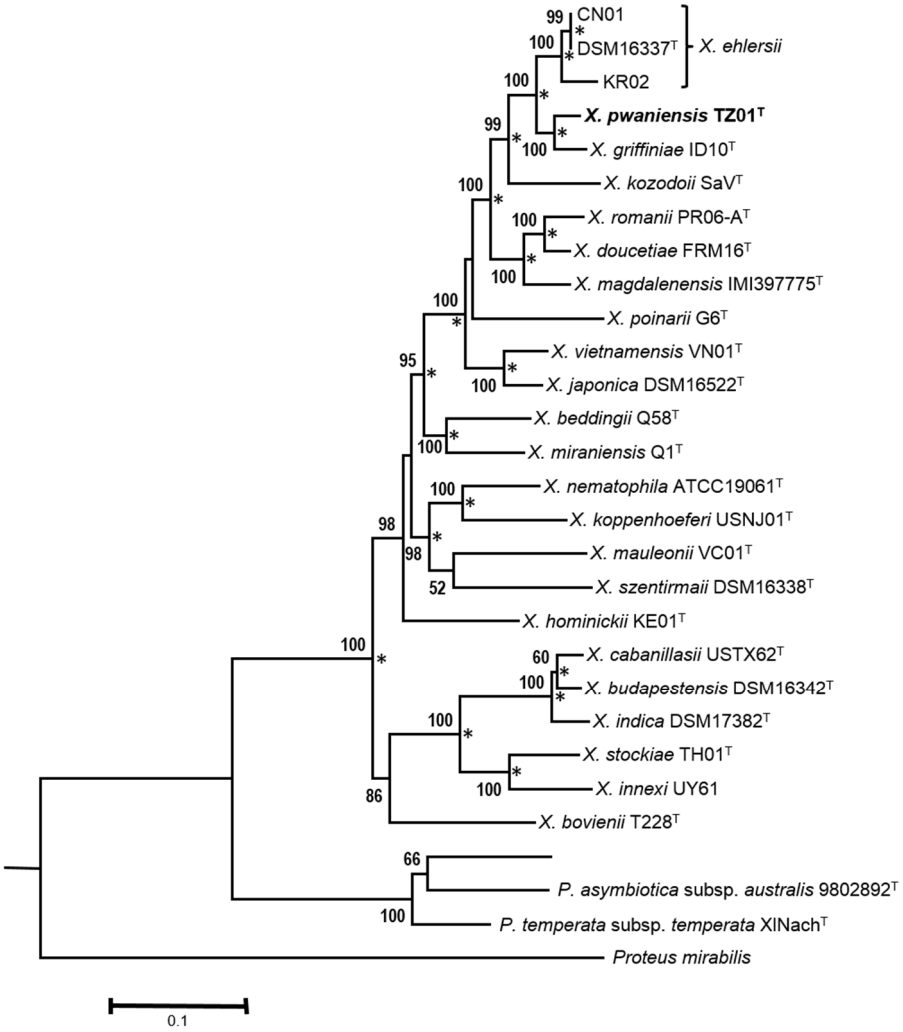

Figure 20: Maximum likelihood phylogenetic tree of *Xenorhabdus* species calculated from five concatenated protein-coding sequences (*recA*, *gyrB*, *dnaN*, *gltX* and *infB*).

The ML analyse was carried out with the General Time Reversible model of substitution with gamma distributed rate heterogeneity and a proportion of invariant sites determined for all the five protein coding sequences by jModelTest to best fit with the data using the AIC criterion (Posada & Crandall, 1998). The concatenated sequences of *Photorhabdus luminescens* subsp. *laumondii* TT01^T, *Photorhabdus temperata*

subsp. *temperata* XlNach^T, *Photorhabdus asymbiotica* subsp. *australis* 9802892^T and *Proteus mirabilis* were used as outgroups. Bootstrap values (Felsentein, 1988) of more than 50% are indicated at the nodes. * indicates the conserved nodes whatever the method of tree reconstruction used (distance, parsimony and ML). Bar represents 10 % divergence.

The values of probability were 0.013 and 0.025 with the codon-based test and the Fisher's exact test, respectively, and can be considered as significant at the level of 5 %. This result suggested that the *recA* sequence of KR02 was under positive selection pressure compared to the other 4 sequences. In the case of the *gltX* gene and considering that CN01 and DSM16337^T have strictly the same sequence, the hypothesis of strict neutrality was also rejected between pairs of sequences for DSM16337 (or CN01), TZ01^T, ID10^T and KR02. Concerning *gyrB*, *dnaN* and *infB*, the hypothesis of strict neutrality was not rejected between pair of sequences for DSM16337^T, CN01 and KR02. However, this hypothesis was rejected between ID10^T and TZ01^T, and between ID10^T (or TZ01^T) and DSM16337^T, CN01 and KR02.

The nucleotide identity between TZ01^T and *X. griffiniae* ID10^T and between TZ01^T and *X. ehlersii* DSM16337^T calculated on the concatenated sequences of the five gene fragments (*recA*, *gyrB*, *dnaN*, *gltX* and *infB*) encompassing 4294 nucleotides is 96.3 % and 95.3 %, respectively which is less than the threshold 97 % used with this approach to delineate *Xenorhabdus* species. Thus, based on this sequence analysis, we propose isolate TZ01^T as a representative of a new *Xenorhabdus* species, *Xenorhabdus pwaniensis* sp. nov.

Table 11: Main phenotypic characters of the previously described *Xenorhabdus* species compared to those of *X. pwaniensis* determined in this study

Species	*X. beddingii*	*X. bovienii*	*X. budapestensis*	*X. cabanillasii*	*X. doucetiae*	*X. ehlersii*	*X. griffiniae*	*X. hominickii*	*X. indica*	*X. innexi*	*X. japonica*	*X. koppenhoeferi*	*X. kozodoii*	*X. magdalenensis*	*X. nuageunii*	*X. miraniensis*	*X. nematophila*	*X. poinarii*	*X. pwaniensis*	*X. romanii*	*X. stockiae*	*X. szentirmaii*	*X. vietnamensis*
Type strain	Q58^T	T228^T	DSM16342^T	USTX62^T	FRM16^T	DSM16337^T	ID10^T	KE01^T	DSM17382^T	DSM16336^T	DSM16522^T	USNJ01^T	SaVT	IMI 397775^T	VC01^T	Q1^T	AN6^T	G6^T	TZ01^T	PR06-A^T	TH01^T	DSM16338^T	VN01^T
Number of strains studied	n = 1	n = 25	n = 2	n = 2	n = 2	n = 5	n = 1	n = 3	n = 2	n = 2	n = 1	n = 1	n = 2	n = 1	n = 1	n = 1	n = 13	n = 5	n = 1	n = 1	n = 1	n = 3	n = 1
Upper threshold temperature for growth	39	32-33	37	39-40	40-42	38-40	39	33-35	39-41	40	34	33	40-41	34	40	38	34-35	40	41	37	39	37-39	37
Pigmentation	+	+	-	V	+	V(+)	-	+	-	V	-	+	+	-	+	+	-	+	+	+	+	V(-)	+
DNase	+	+	+	+	V	V(-)	+	+	+	+	+	+	-	+	+	-	+	V(-)	+	-	+	+	+
Simmons' citrate	+	V(-)	-	-	-	V(-)	+	-	-	V	-	-	V	-	+	+	V(-)	V(-)	-	-	-	V(+)	-
Aesculin hydrolysis	+	-	V	-	V	V(+)	+	+	V	+	-	-	V	-	+	+	V(-)	V(-)	V	-	+	+	+
Lecithinase	+	V(+)	V	V	+	V(+)	+	+	V	+	-	+	+	-	+	+	V(+)	-	+	-	+	-	+
Acid production from:																							
Ribose	+	V(-)	-	-	V	V(+)	+	V(-)	-	+	+	-	+	-	+	+	V(-)	-	+	-	+	V(-)	-
Glucose	+	+	+	+	V	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	V(+)	+	V	V	V(+)	+	+	+	+	+	-	-	-	+	+	+	V(+)	-	+	+	V(+)	+
Mannose	+	+	+	+	V	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	V(+)	+

Inositol	-	V(-)	+	V	V	-	-	V(-)	+	V	-	-	-	-	+	-	V(-)	-	-	w	+	V(+)	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V(+)	-
N-acetyl glucosamine	+	+	V	+	V	V(+)	-	V(+)	V	+	+	-	+	-	+	+	+	+	+	+	+	V(+)	+
Esculine	+	-	-	-	V	V(+)	+	+	V	-	-	-	+	+	+	+	-	V(-)	-	-	+	+	+
Maltose	+	V(+)	-	V	V	V(+)	-	V(+)	-	-	+	+	+	+	+	+	V(+)	+	-	+	+	+	+
Trehalose	+	V(+)	-	-	+	-	-	V(-)	-	V	-	-	+	+	+	+	V(+)	V(+)	-	+	-	-	+
Gluconate	+	V(-)	-	-	-	-	-	-	-	+	-	-	+	-	+	w	-	-	-	w	+	-	-
5-Keto gluconate	+	V(-)	+	V	-	V(+)	w	+	+	+	+	+	-	-	w	w	V(+)	V(-)	-	w	w	-	+
Assimilation of:																							
Glycerol	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Ribose	+	V(+)	-	-	V	V(-)	-	V(-)	-	+	-	-	+	-	+	+	-	-	+	-	+	V(+)	-
Glucose	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	V(+)	+	V	V	V(-)	-	V(+)	+	+	+	-	-	-	+	+	+	V(+)	-	+	V(+)	-	+
D-mannose	+	+	+	+	V	V(+)	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Inositol	-	-	-	-	+	-	w	V(-)	+	+	+	-	-	-	+	w	V(+)	-	-	+	+	+	w
N-acetyl glucosamine	+	+	V	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Esculine	+	-	V	-	V	V(+)	+	+	V	V	-	-	V	+	+	+	-	V(+)	-	-	+	+	+
D-Maltose	+	+	V	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
D-trehalose	+	+	+	V	+	V(+)	+	+	V	+	+	-	+	+	+	+	+	+	+	+	-	V(+)	+
D-Gluconate	+	+	V	-	+	V(+)	+	+	+	+	w	-	V	-	+	+	V(-)	+	+	+	+	+	+

2

3 +, 90% of strains positive; V(+), 50 to 89% of strains positive; V(-), 11 to 49% of strains positive; -, 0 to 10% of strains positive; V, variable; w, weak

4.3. Phenotypic characterization of isolate TZ01^T.

Isolate TZ01^T was Gram negative, had no catalase and nitrate reductase activities, two characters which allow differentiation of the genus *Xenorhabdus* from other genera of the family Enterobacteriaceae (Akhurst & Boemare, 2005). Colonies of TZ01^T studied had the typical phenotypic traits of *Xenorhabdus* primary form variants isolated from the intestine of infective-stage nematodes (Boemare & Akhurst, 1988): colonies were pigmented (yellow), they absorbed dyes from NBTA, produced antimicrobial compounds detected using *Micrococcus luteus* as indicator organism, and had lecithinase activities. Isolate TZ01^T had an upper temperature limiting growth of 41°C comparable with those of other sister species *X. griffiniae* (39°C), *X. ehlersii* (38-40°C) and *X. kozodoii* (40-41°C). However, isolate TZ01^T can be differentiated from each of these three sister species by several phenotypic traits (Table 11).

4.4. Fatty acid analysis of *X. ehlersii*, *X. griffiniae* and isolate TZ01^T.

The analysis of fatty acid composition of the 3 *Xenorhabdus* species studied showed six major fatty acids (14:0 - 14:0 3OH/16:1 iso I - 16:1 w7c/16:1 w6c - 16:0 - 17:0 cyclo - 18:1 w7c) which represented each, 5 % to 30 % of the total amount of fatty acids detected (Figure S7A, supplementary data). Twenty four minor fatty acids were also detected and represented for each, less than 2% of the total amount (Figure S7B, supplementary data). The PLS discriminate analysis allowed to distinguish clearly strains of the three *Xenorhabdus* species tested (Figure 21A) and to propose a combination of fatty acids which characterized each species (Figure 21B). Thus, *X. pwaniensis* TZ01^T was

characterized by significant higher amounts of minor fatty acids 17:0 and 18:0. The proportion of major fatty acid 18:1 w7c was also significantly higher for *X. pwaniensis* TZ01^T than for *X. griffiniae* ID10^T. *X. griffiniae* ID10^T was characterized by significantly higher proportion of major fatty acids 16:0 and 17:0 cyclo and minor fatty acids 12:0 3OH and 19:0 cyclo. At least, *X. ehlersii* strains were characterized by significantly higher proportion of major fatty acid 14:0 and minor fatty acid 17:1 w9c. Janse & Smits (1990) showed that the two closely related genera *Photorhabdus* and *Xenorhabdus* could be clearly differentiated by their fatty acid composition. We showed here that this approach allowed to distinguish three closely related species within the genus *Xenorhabdus*.

4.5. Pathogenicity of isolate TZ01^T.

Isolate TZ01^T was highly pathogenic for *G. mellonella* and *S. littoralis* larvae (Table 12) following intrahaemocoelic injection. All the *G. mellonella* larvae infected with 10^2 cells of TZ01^T were killed within 24h. All the *S. littoralis* larvae infected with 10^3 cells of TZ01^T were killed within 28h. In contrast, no mortality occurred within 48h when 10^6 non pathogenic *E. coli* cells were injected in the larvae. For *X. poinarii*, which is characterized by its low pathogenicity when it is injected alone without its nematode host (Akhurst, 1986), no mortality was observed with *Spodoptera* larvae and 75% mortality was reached with the susceptible insect *G. mellonella* within 24h with 10^4 bacterial cells injected intrahaemocoelically.

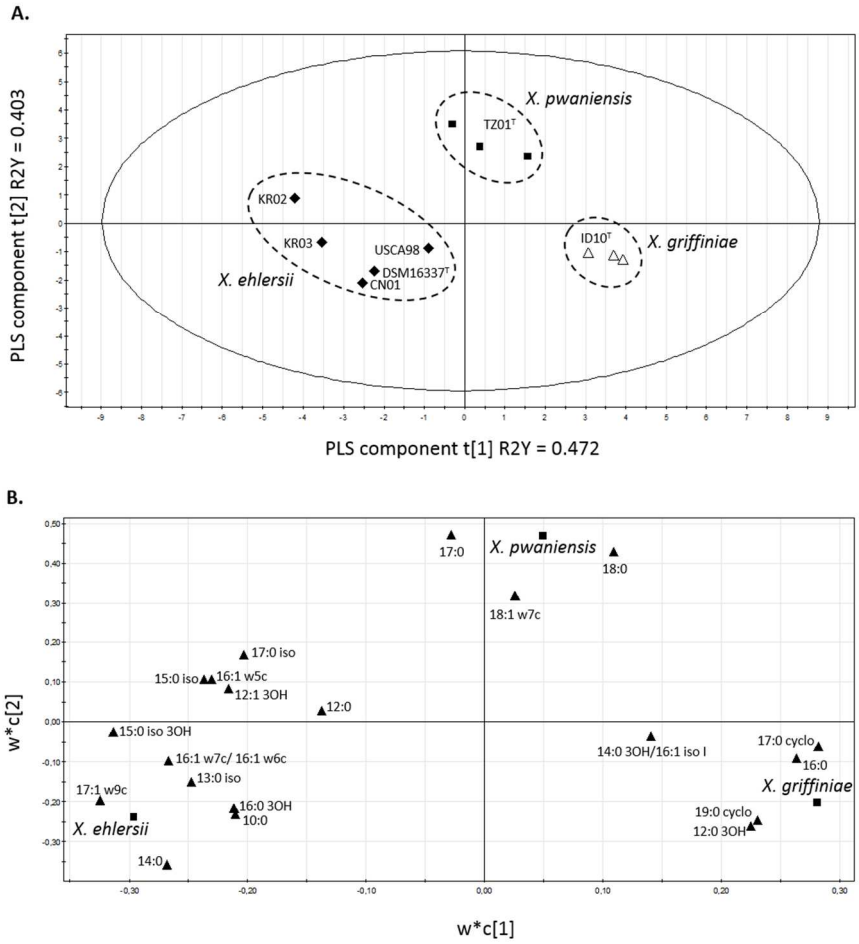

Figure 21: ***Xenorhabdus*** **discrimination based on fatty acids.**

A. Partial least square-discriminate analysis based on fatty acid composition between *X. pwaniensis* TZ01^T (3 replicates represented by black boxes), *X. griffiniae* ID10^T (3 replicates represented by open triangles) and *X. ehlerii* (5 strains represented by black lozanges) grouped in dashed lines, respectively. The cross-validation leads to 2 PLS components. The corresponding PLS model explains 87.5 % (sum of R2Y) of the variation of the Y-matrix. The 95% probability region defined by the model is delimited by the ellipse.

B. Plot showing the X-loadings (w*) corresponding to the fatty acids represented by black triangles in relationship with the Y-loadings (c) corresponding to the three *Xenorhabdus* species studied and represented by black boxes. The three *Xenorhabdus* species were clearly differentiated by a combination of several fatty acids.

Table 12: Mortality of *G. mellonella* and *S. littoralis* larvae after intrahaemocoelic injection of *Xenorhabdus* sp. and *E. coli* bacterial cells.

	G. mellonella	*S. littoralis*
E. coli	10^6 cells injected no mortality 48h after injection	10^6 cells injected no mortality 48h after injection
X. poinarii strain G6 ^{T}	10^4 cells injected 75% mortality 24h after injection	10^4 cells injected no mortality 24h after injection
X. pwaniensis strain TZ01^T	10^2 cells injected 100% mortality in less than 24h	10^3 cells injected 100% mortality in less than 28h
X. nematophila strain F1	10^2 cells injected 100% mortality in less than 21h	10^2 cells injected 90% mortality 30h after injection

5. Description of *Xenorhabdus pwaniensis* sp. nov.

Xenorhabdus pwaniensis [pwa.ni.en'sis N.L. fem. adj. *pwaniensis* from Pwani (meaning "coast" in Swahili language), a coastal region of East Tanzania, the source of the nematode from which the type strain was isolated].

X. pwaniensis is a Gram negative bacterium which is not able to reduce nitrate and has no catalase activity. Growth in LB broth is stopped by temperatures above 41°C. Colonies are yellow pigmented and show DNase and lecithinase activities. Aesculin hydrolysis is variable.

X. pwaniensis can be differentiated from *X. griffiniae* by its capability to produce acid from glucose, mannose and N-acetyl glucosamine. Tests are negative for Simmons' citrate.

X. pwaniensis can be differentiated from the majority of the *X. ehlersii* strains tested in this study by its DNAse activity and the absence of acid production from maltose and 5-Keto gluconate.

In contrast to its both sister species, *X. pwaniensis* is able to assimilate ribose but not esculine and is not able to produce acid from fructose and esculine.

The type strain is TZ01^T (=DSM25309^T, = CIP110340^T). The GenBank accession numbers of the type strain are JQ687358 (16S rRNA gene), JQ687369 (*recA*), JQ687370 (*gyrB*), JQ687371 (*dnaN*), JQ687372 (gltX), JQ687373 (*infB*).

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7. Supplementary data

Description of *Xenorhabdus tanzaniensis* sp. nov., the symbiotic bacterium associated with a new *Steinernema* species closely related to *S. karii and* isolated in Tanzania

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Summary

The symbiotic bacterium (TZ01^T) was isolated from a new species of insect-pathogenic nematode of the genus *Steinernema*. On the basis of 16S rRNA gene sequence similarity, this bacterial isolate was shown to belong to the genus *Xenorhabdus* in agreement with the genus of its nematode-host. The accurate phylogenetic position of this new isolate was defined using a multigene approach and showed that isolate TZ01^T shares a common ancestor with *X. griffiniae* ID10^T, *X. ehlersii* DSMZ16377^T and *X. kozodoii* SaV^T, the symbiotic bacteria associated with *Steinernema hermaphroditum*, *Steinernema longicaudum*

and *Steinernema arenarium* respectively. The nucleotide identity (less than or equal to 96.1%) between TZ01^T, *X. griffiniae* ID10^T, *X. ehlersii* DSMZ16377^T and *X. kozodoii* SaV^T calculated on the concatenated sequences of five gene fragments encompassing 4286 nucleotides and the difference between these four bacteria allowed genetic and phenotypic differentiation of isolate TZ01^T from its three closely related species. TZ01^T therefore represents a new species, for which the name *Xenorhabdus tanzaniensis* sp. nov. is proposed, with the type strain DSM25309^T (=CIP110340^T).

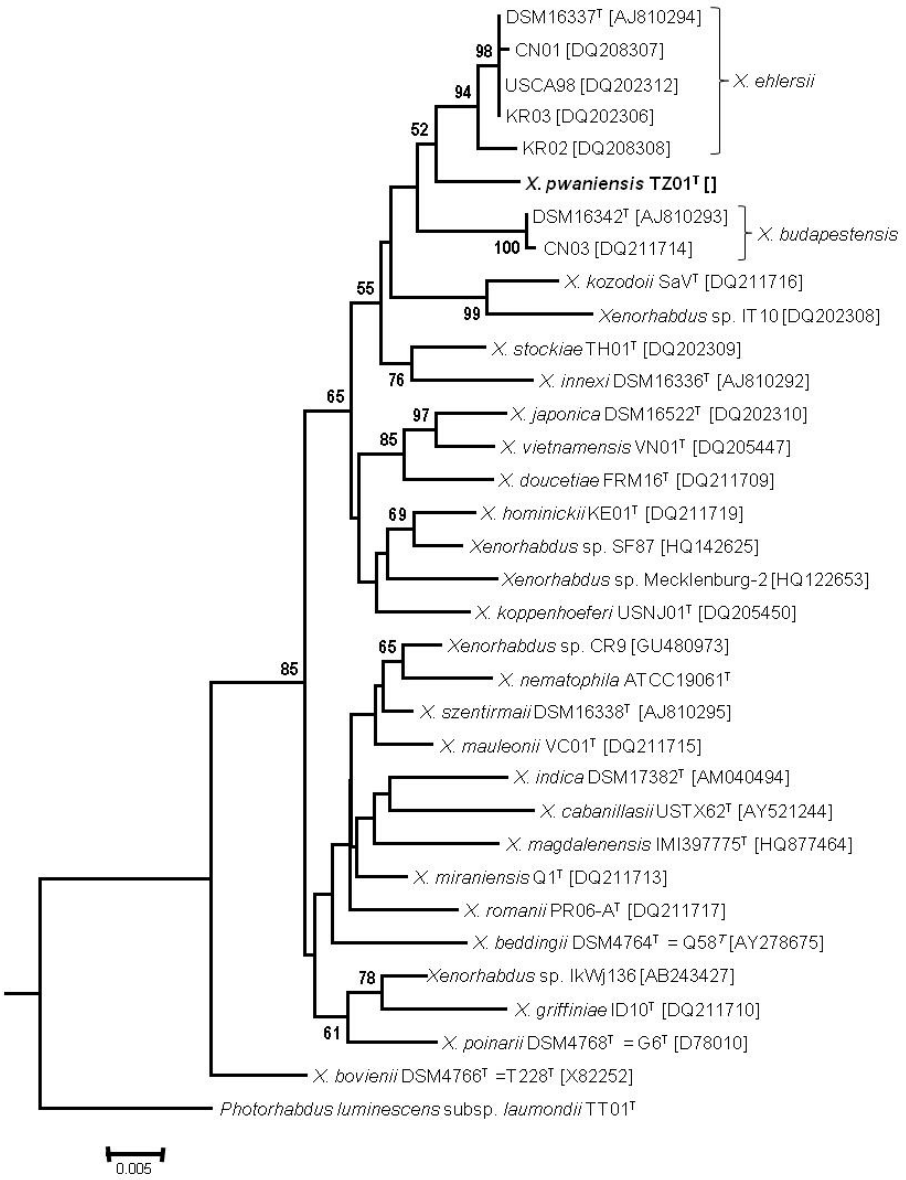

Figure S1: Distance tree based on 16S rDNA sequences of *Xenorhabdus* type strains and representatives of potential new species including *X. pwaniensis* TZ01T. The tree was constructed using the Kimura 2-parameter model (Kimura, 1980) and the neighbour-joining module (Saitou & Nei, 1987) present on the PAUP software (Swofford, 2003). Bootstrap values (Felsentein, 1988) of more than 50% are indicated at the nodes. Bar indicates 0.5 % sequence divergence. GenBank accession numbers of the sequences are in brackets. The sequences of *X. nematophila* ATCC19061T and *P. luminescens* subsp. *laumondii* TT01T were from

http://www.cns.fr/agc/microscope/home/index.php. The 16S rDNA sequences of *X. ehlersii* DSM16337^T and *X. pwaniensis* TZ01^T share 98.6 % nucleotide identity on a length of 1319 nucleotides.

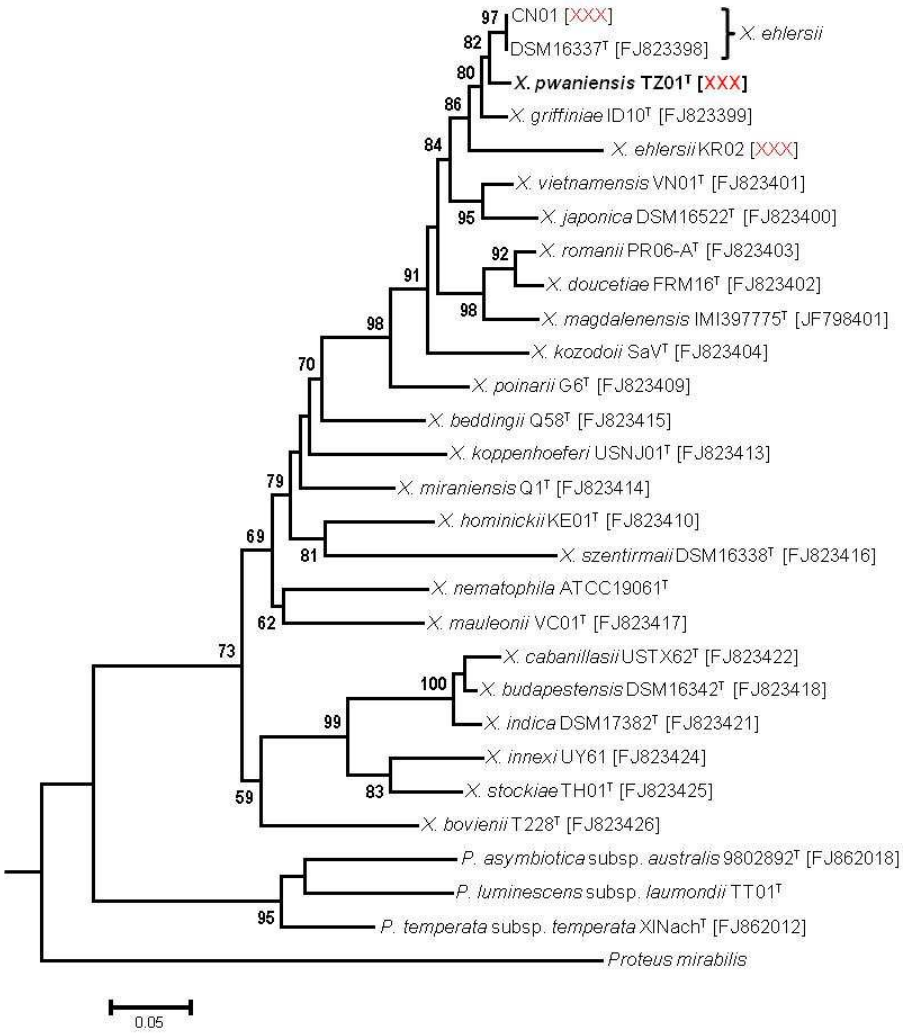

Figure S2: ML tree based on *recA* sequences of *Xenorhabdus* type strains including *X. pwaniensis* TZ01^T. The ML analyse was carried out with the GTR model of substitution with gamma distributed rate heterogeneity and a proportion of invariant sites determined jModelTest to best fit with the data using the AIC criterion (Posada & Crandall, 1998). The sequences of *Photorhabdus luminescens* subsp. *laumondii* TT01^T, *Photorhabdus temperata* subsp. *temperata* XlNach^T, *Photorhabdus asymbiotica* subsp. *australis* 9802892^T and *Proteus mirabilis* were used as outgroups. Values (>50 %) of the approximate likelihood ratio test (Anisimova & Gascuel, 2006) are shown at the node.

Bar represents 10 % divergence. GenBank accession numbers of the sequences are in brackets. The sequences of *X. nematophila* ATCC19061^T and *P. luminescens* subsp. *laumondii* TT01^T were from http://www.cns.fr/agc/microscope/home/index.php. The sequence of *P. mirabilis* was from GenBank [NC010554]

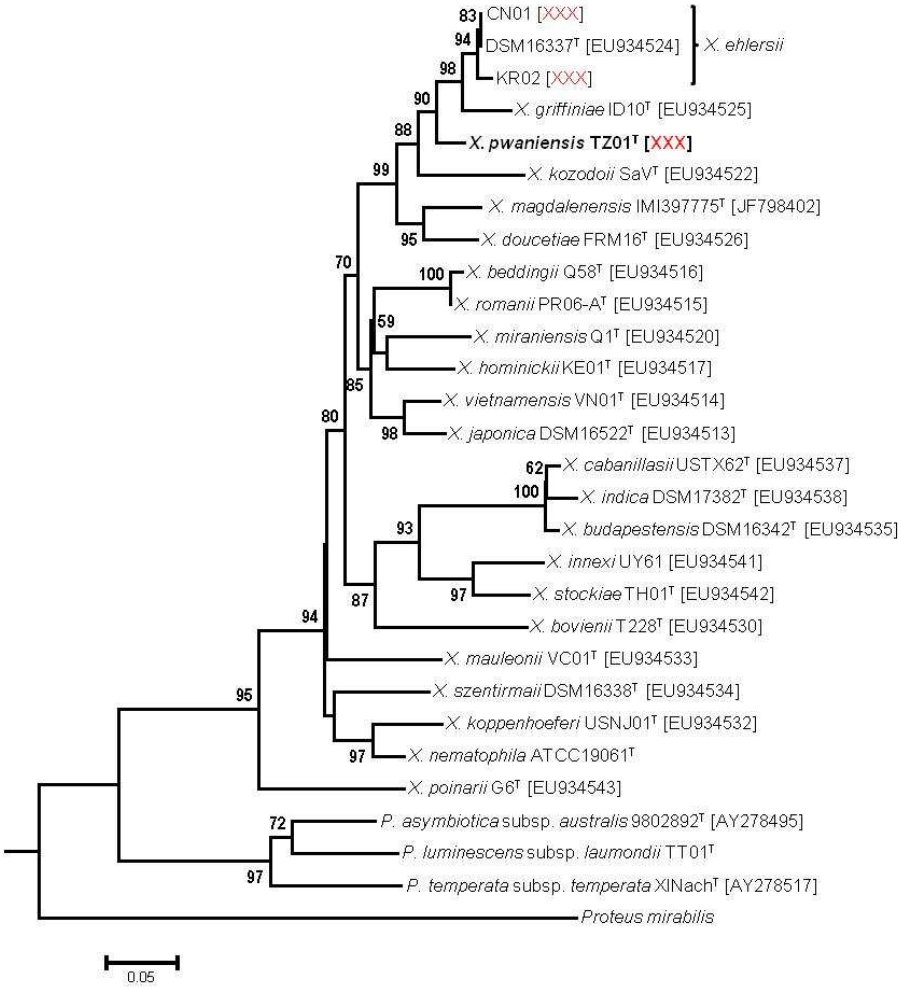

Figure S3: ML tree based on *gyrB* sequences of *Xenorhabdus* type strains including *X. pwaniensis* TZ01^T. The ML analyse was carried out with the GTR model of substitution with gamma distributed rate heterogeneity and a proportion of invariant sites determined jModelTest to best fit with the data using the AIC criterion (Posada & Crandall, 1998). The sequences of *Photorhabdus luminescens* subsp. *laumondii* TT01^T, *Photorhabdus temperata* subsp. *temperata* XINach^T, *Photorhabdus asymbiotica* subsp. *australis* 9802892^T and *Proteus mirabilis* were used as outgroups. Values (>50 %) of the approximate likelihood ratio test (Anisimova & Gascuel, 2006) are shown at the node. Bar represents 10 % divergence. GenBank accession numbers of the sequences are in

brackets. The sequences of *X. nematophila* ATCC19061^T and *P. luminescens* subsp. *laumondii* TT01^T were from http://www.cns.fr/agc/microscope/home/index.php. The sequence of *P. mirabilis* was from GenBank [NC010554].

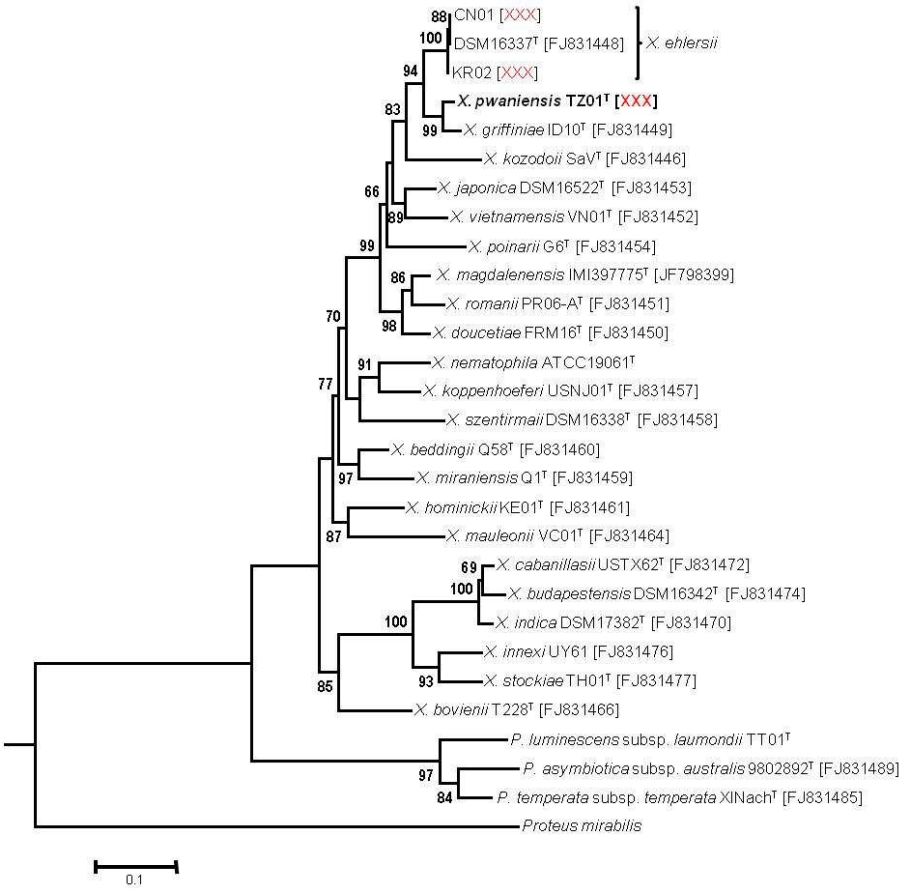

Figure S4: ML tree based on *dnaN* sequences of *Xenorhabdus* type strains including *X. pwaniensis* TZ01^T. The ML analyse was carried out with the GTR model of substitution with gamma distributed rate heterogeneity and a proportion of invariant sites determined jModelTest to best fit with the data using the AIC criterion (Posada & Crandall, 1998). The sequences of *Photorhabdus luminescens* subsp. *laumondii* TT01^T, *Photorhabdus temperata* subsp. *temperata* XlNach^T, *Photorhabdus asymbiotica* subsp. *australis* 9802892^T and *Proteus mirabilis* were used as outgroups. Values (>50 %) of the approximate likelihood ratio test (Anisimova & Gascuel, 2006) are shown at the node. Bar represents 10 % divergence. GenBank accession numbers of the sequences are in brackets. The sequences of *X. nematophila* ATCC19061^T and *P. luminescens* subsp. *laumondii* TT01^T were from http://www.cns.fr/agc/microscope/home/index.php. The sequence of *P. mirabilis* was from GenBank [NC010554].

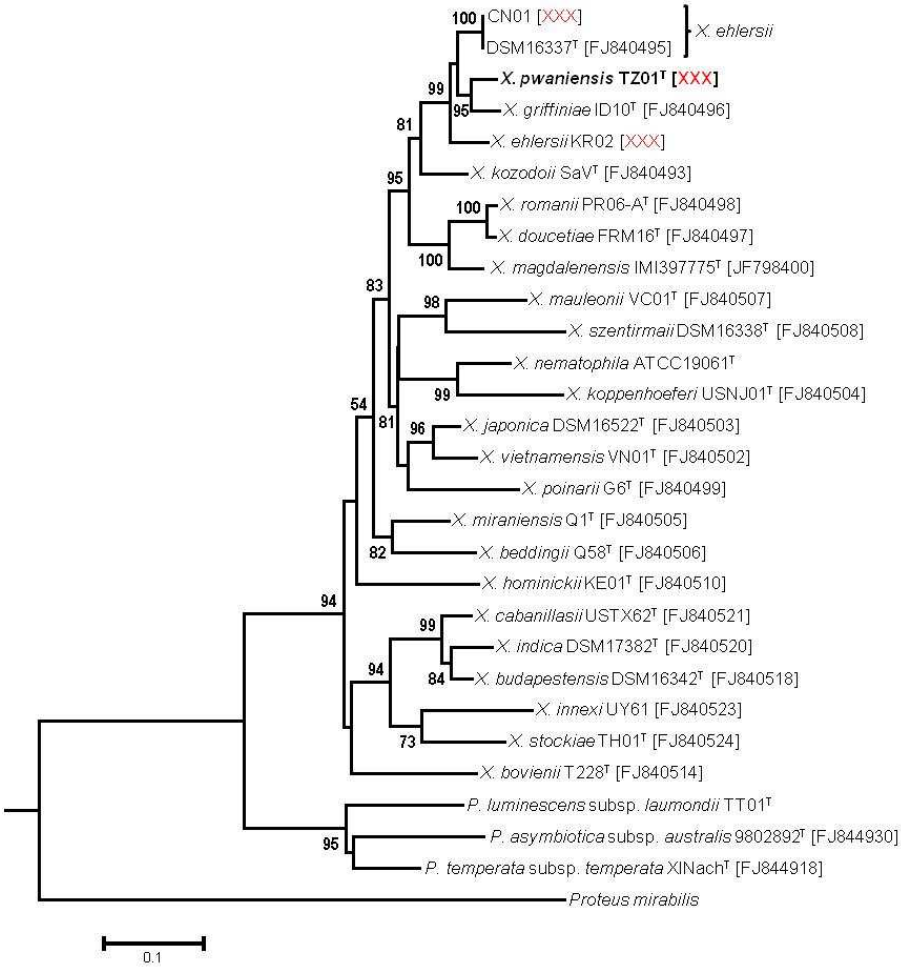

Figure S5: ML tree based on *gltX* sequences of *Xenorhabdus* type strains including *X. pwaniensis* TZ01T. The ML analyse was carried out with the GTR model of substitution with gamma distributed rate heterogeneity and a proportion of invariant sites determined jModelTest to best fit with the data using the AIC criterion (Posada & Crandall, 1998). The sequences of *Photorhabdus luminescens* subsp. *laumondii* TT01T, *Photorhabdus temperata* subsp. *temperata* XlNachT, *Photorhabdus asymbiotica* subsp. *australis* 9802892T and *Proteus mirabilis* were used as outgroups. Values (>50 %) of the approximate likelihood ratio test (Anisimova & Gascuel, 2006) are shown at the node. Bar represents 10 % divergence. GenBank accession numbers of the sequences are in brackets. The sequences of *X. nematophila* ATCC19061T and *P. luminescens* subsp. *laumondii* TT01T were from http://www.cns.fr/agc/microscope/home/index.php. The sequence of *P. mirabilis* was from GenBank [NC010554].

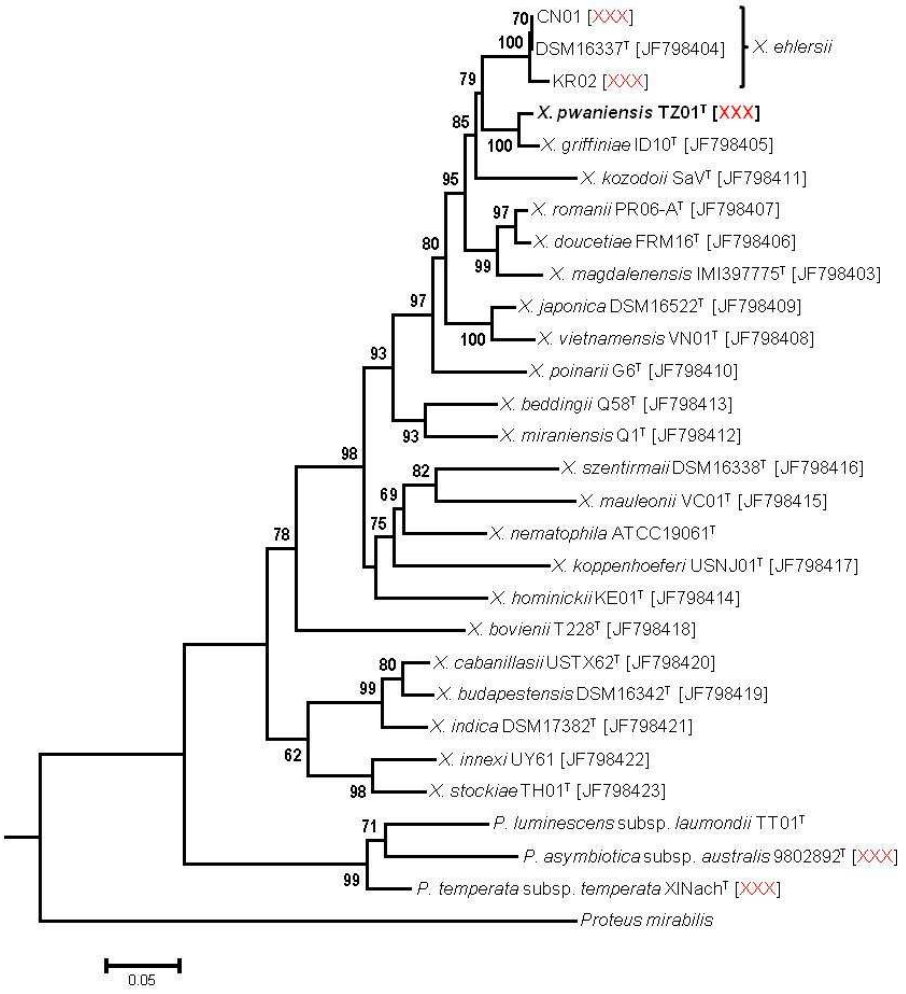

Figure S6: ML tree based on *infB* sequences of *Xenorhabdus* type strains including *X. pwaniensis* TZ01T. The ML analyse was carried out with the GTR model of substitution with gamma distributed rate heterogeneity and a proportion of invariant sites determined jModelTest to best fit with the data using the AIC criterion (Posada & Crandall, 1998). The sequences of *Photorhabdus luminescens* subsp. *laumondii* TT01T, *Photorhabdus temperata* subsp. *temperata* XlNachT, *Photorhabdus asymbiotica* subsp. *australis* 9802892T and *Proteus mirabilis* were used as outgroups. Values (>50 %) of the approximate likelihood ratio test (Anisimova & Gascuel, 2006) are shown at the node. Bar represents 10 % divergence. GenBank accession numbers of the sequences are in brackets. The sequences of *X. nematophila* ATCC19061T and *P. luminescens* subsp. *laumondii* TT01T were from http://www.cns.fr/agc/microscope/home/index.php. The sequence of *P. mirabilis* was from GenBank [NC010554].

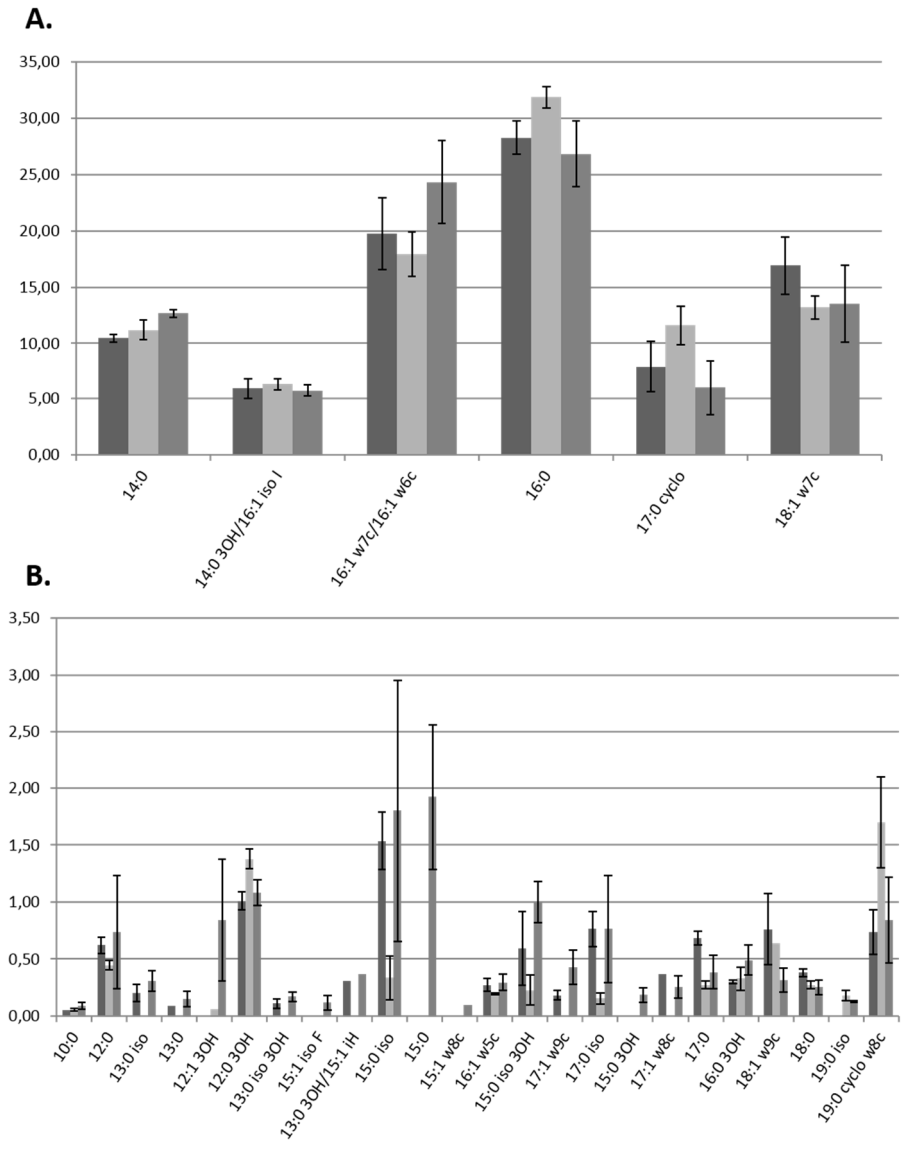

Figure S7: Fatty acid composition for *X. pwaniensis* TZ01^T and closely related *Xenorhabdus* species. Mean (%) of fatty acids and standard deviation for *X. pwaniensis* (■ - 3 replicates, 1 strain), *X. griffiniae* (■ - 3 replicates, 1 strain) and *X. ehlersii* (■ - 1 replicate, 5 strains). **A.** Major fatty acids (> 5 % of the total). **B.** Minor fatty acids (< 2 % of the total).

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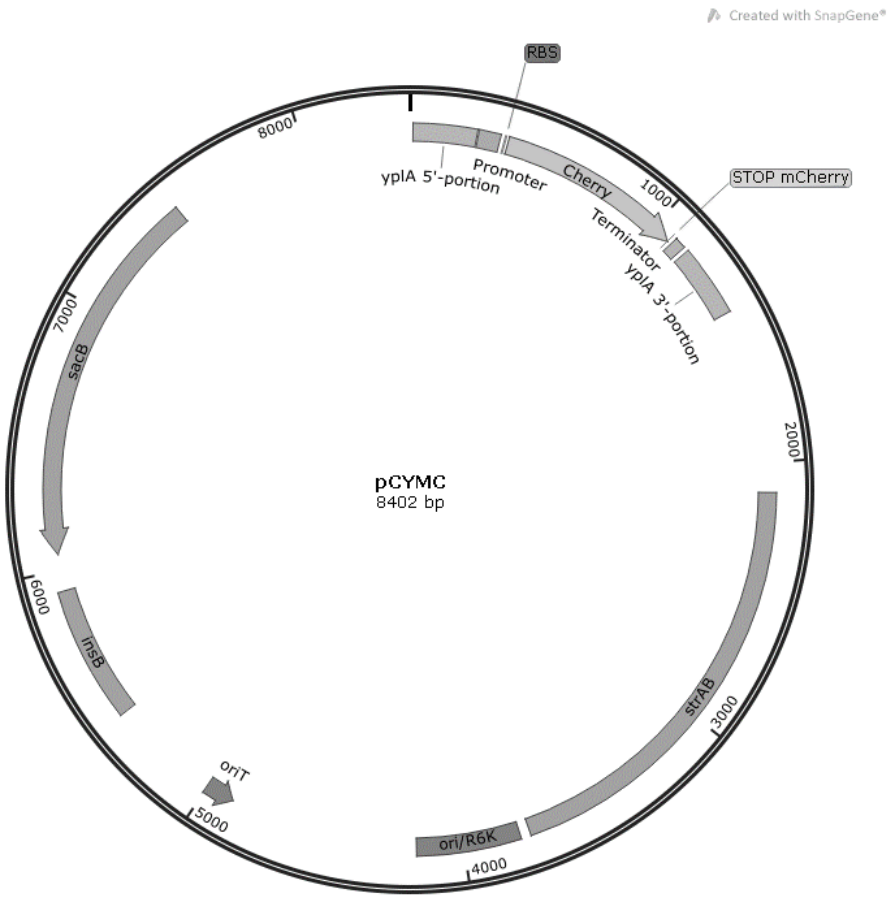
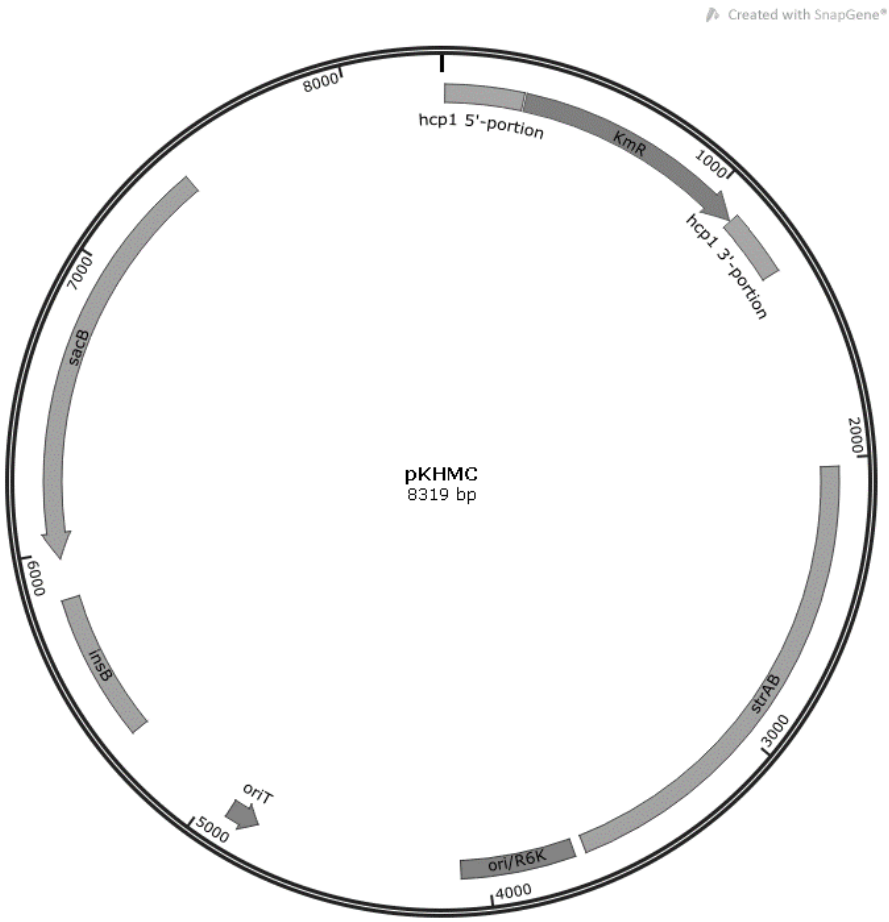
Appendix III: Plasmid maps

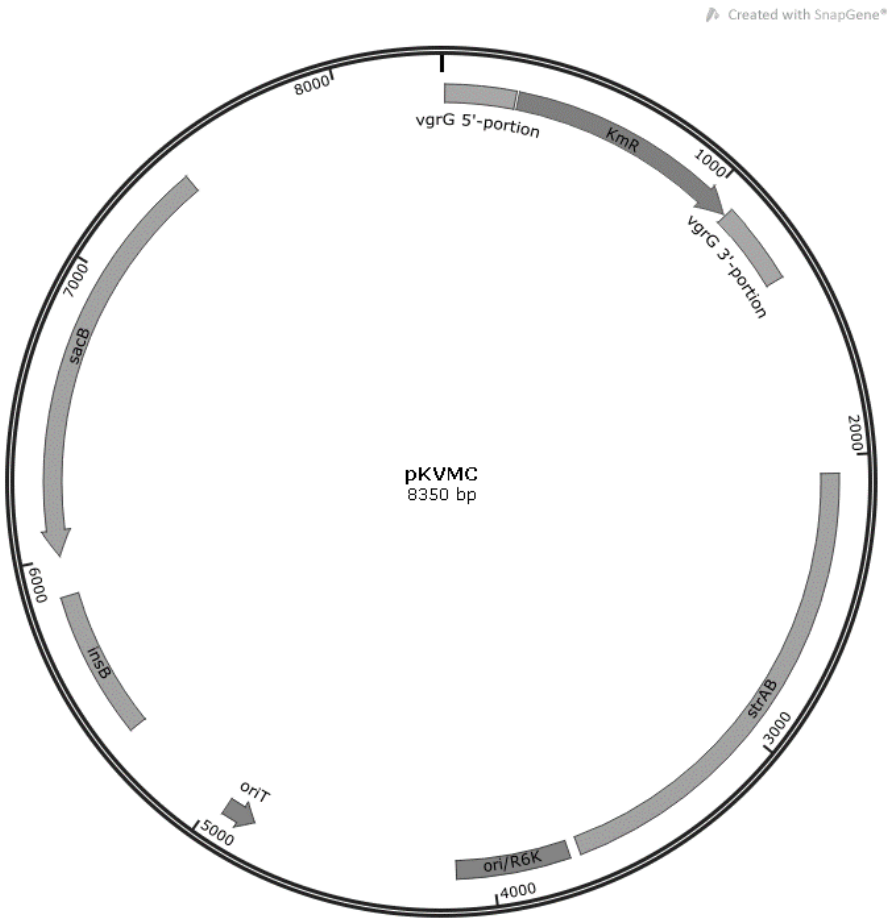
Mutator plasmid for *yplA*:



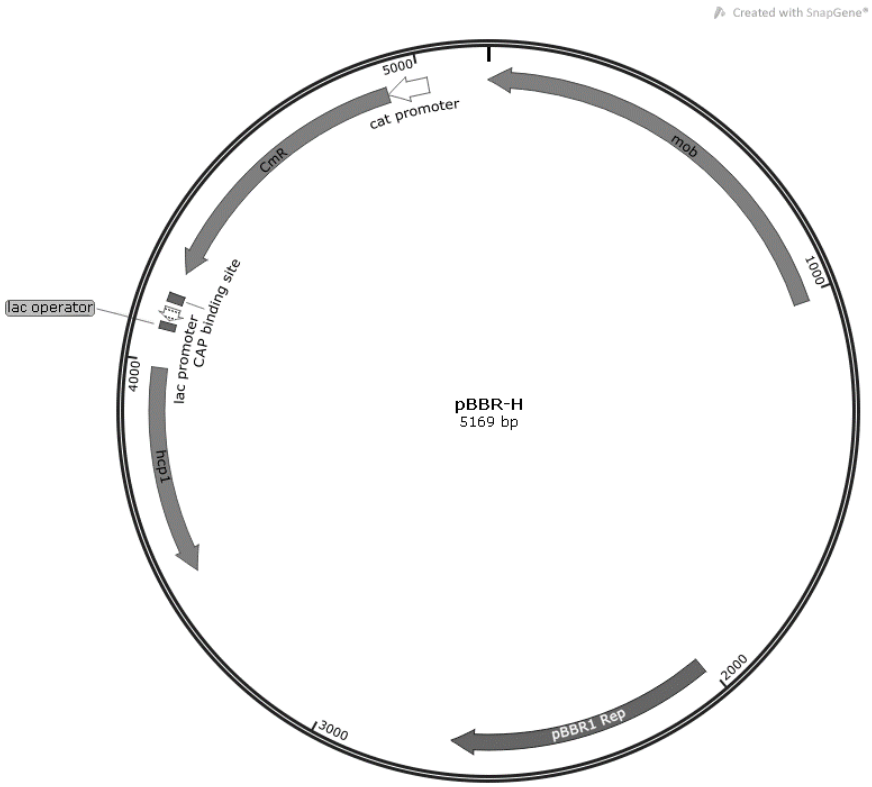
Mutator plasmid for *hcp1*:



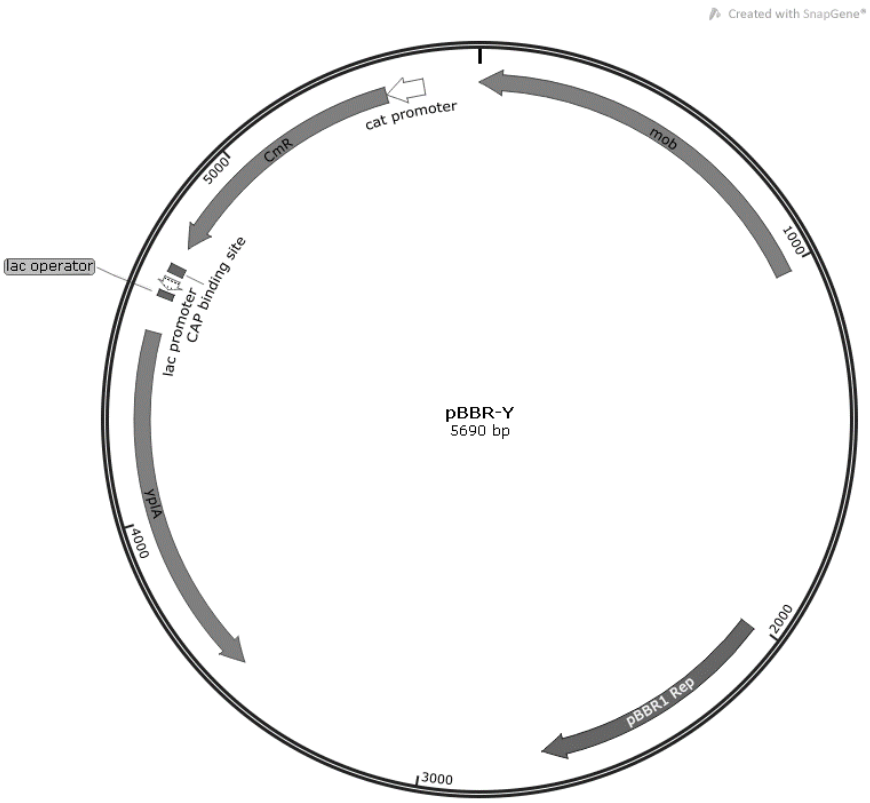
Mutator plasmid for *vgrG*:



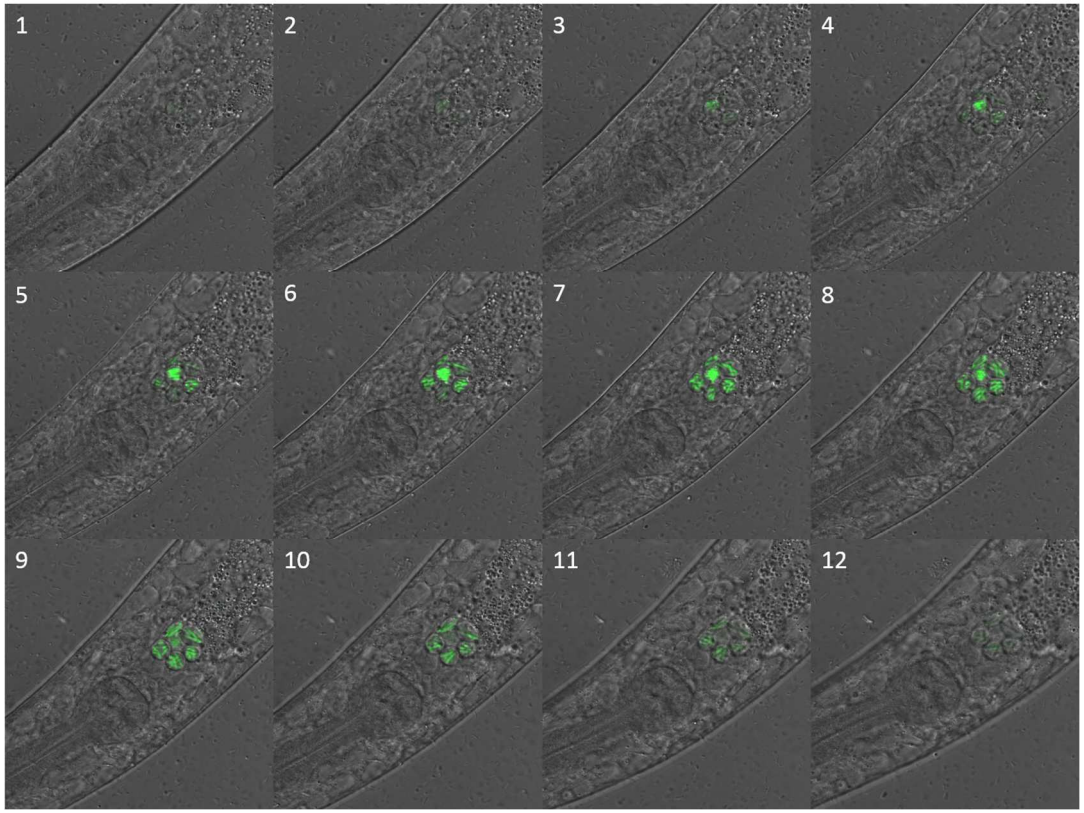
Complementation vector for *hcp1*:



Complementation vector for *yplA*:



Appendix IV: *Steinernema* receptacle segregation



Confocal microscope slides in Z-axis (numbered from 1 to 12) of the anterior partion of a *Steinernema* sp. MW8B male colonized by Xenorhabdus sp. TZ03 (GFP-labelled).

6 subdivisions of the receptacle appear clearly where *X.* sp. TZ03 locates.